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**JOANA BRAGA DE MORAES MARQUES FERREIRA**

# TRIAGEM DE GENES RELACIONADOS AO METABOLISMO DO FOSFATO INORGÂNICO EM FAMÍLIAS COM CALCIFICAÇÃO IDIOPÁTICA DOS NÚCLEOS DA BASE DO CÉREBRO (IBGC)

RECIFE

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do grau de Doutora em Ciências Biológicas.

Orientador: Prof. Dr. João Ricardo Mendes de Oliveira

Recife
2016

JOANA BRAGA DE MORAES MARQUES FERREIRA

# SCREENING OF GENES RELATED TO INORGANIC PHOSPHATE IN FAMILIES WITH PRIMARY BRAIN CALCIFICATIONS (PBC)

Doctorate thesis presented to the Graduate Program in Biological Sciences of the Federal University of Pernambuco, as required for achieving the degree in Biological Sciences Doctor.

Advisor: Prof. Dr. João Ricardo Mendes de Oliveira

Recife

2016

Catalogação na fonte
Elaine Barroso
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Recife

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*A todas as pessoas que considero família,*
*este trabalho também é de vocês.*

*To everyone that is like family to me,*
*this work is yours too.*

## ABSTRACT

Primary brain calcification (PBC), also known as idiopathic brain calcification or Fahr's disease, is a rare neurological condition that is characterized by calcium phosphate deposits in the basal ganglia and adjacent areas, movement disorders, headache and neuropsychiatric symptoms. It presents autosomic dominant inheritance and it is associated with two inorganic phosphate transporter coding genes: *SLC20A2* and *XPR1*. Two other genes related to the blood-brain barrier maintenance and integrity are also linked to PBC, the platelet-derived growth factor-β and its receptor (*PDGFB* and *PDGFRB*), although their roles in the formation mechanism of the calcifications is not clear yet. For this study, besides the four genes above mentioned, other members of the platelet-derived grown factor family (*PDGFA*, *PDGFRA*, *PDGFC* and *PDGFD*) have also been selected as candidate genes, for which new primer pairs were designed. All genes above were screened for new variants by Sanger sequencing in fifteen Brazilian unrelated patients with brain calcifications. Sequence *in silico* analysis was performed using CLC Main Workbench 6.9 software and online tools available in NCBI and GOLDENPATH platforms, resulting in the identification of the first *de novo* *SLC20A2* mutation in a patient diagnosed with PBC (NM_006749.4:c.1158C>G; NP_006740.1:p.Y386*). *SLC20A2* is to-date the main gene associated with PBC, with affecting-variants observed in ~50% cases. In order to find *SLC20A2* deletions and/or duplications not detected by sequencing, all Brazilian probands were screened by QMPSF (Quantitative Multiplex PCR of Short fluorescent Fragments) and a duplication of the terminal exon was found in a patient with brain calcifications and hyperparatiroidism. Simultaneously, twenty-four French unrelated patients with PBC were also analyzed by QMPSF and partial *SLC20A2* deletions were detected in four patients: two with deletion of the exon 2, where the start codon is located; one with deletion of the exon 4; and one with deletion of exons 4 and 5. These results reinforce *SLC20A2* role as the main gene associated to PBC, as well as demonstrate that copy number variation analyses, even when revealing only partial deletions or duplications of a gene, are complementary to sequencing and work side by side in the search of genetic variations involved in this disease.



**Key-words:** primary brain calcifications, Fahr's disease, sequencing, selection of candidate genes, inorganic phosphate.

## RESUMO

**Introdução:** A calcificação cerebral primária (CCP), também conhecida como calcificação idiopática dos núcleos da base ou doença de Fahr, é uma condição neurológica caracterizada por depósitos de fosfato de cálcio dos núcleos da base e região de entorno, parkinsonismo e sintomas neuropsiquiátricos. Apresenta herança autossômica dominante e é associada a dois genes codificantes de transportadores de fosfato inorgânico: o *SLC20A2* e o *XPR1*. Dois outros genes relacionados à manutenção e à integridade da barreira hemato-encefálica, o fator de crescimento plaquetário B e seu receptor (*PDGFB* e *PDGFRB*), também foram associados à CCP, embora seus papeis no mecanismo de formação das calcificações ainda não estejam claros. **Materiais e Métodos:** Além dos quatro genes acima, foram selecionados como candidatos outros genes da família dos fatores de crescimento plaquetário (*PDGFA*, *PDGFRA*, *PDGFC* e *PDGFD*) e das protocaderinas (*PCDH12*), para os quais foram confeccionados pares de *primers* utilizados no seu sequenciamento e para análise de variação de número de cópia. **Resultados e Discussão:** Quinze famílias brasileiras com CCP foram triadas para novas variantes nos genes candidatos por sequenciamento. A análise *in silico* do sequenciamento foi feita através do *software* CLC Combined Workbench versão 6.9 e das ferramentas disponíveis nas plataformas *online* do NCBI e do GOLDENPATH. A partir dessa análise, foi identificada em um probando a primeira mutação de novo do *SLC20A2*, o principal gene associado a CCP (NM_006749.4:c.1158C>G; NP_006740.1:p.Y386*). A fim de encontrar deleções e/ou duplicações do *SLC20A2* não detectadas por sequenciamento, todos os probandos brasileiros com calcificações cerebrais foram triados através da técnica de QMPSF (do inglês, *Quantitative Multiplex PCR of Short fluorescent Fragments*). Foi encontrada uma duplicação do exon terminal do mesmo gene em um paciente brasileiro com calcificações cerebrais e hiperparatireoidismo. Simultaneamente, foram identificadas deleções parciais no mesmo gene em quatro famílias francesas com CCP. **Conclusões:** Esses resultados reafirmam o *SLC20A2* como o principal gene associado a CCP, bem como demonstram que análises de variação de número de cópia (CNV), ainda que parciais, são complementares ao sequenciamento na busca por variantes genéticas relacionadas a esta doença.



**Palavras chaves:** Calcificações cerebrais primárias, Doença de Fahr, Sequenciamento, Seleção de Genes Candidatos, Fosfato inorgânico.

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# 1. INTRODUCTION

Primary Brain Calcification (PBC), also known as Fahr's Disease, is a rare condition characterized by bilateral calcifications in the Brain with an autosomal dominant pattern of heritance. According to Legati et al. (2015), "PFBC symptoms typically occur after the age of 40, with progressive neuropsychiatric and movement disorders, although some individuals may remain asymptomatic." Several publications have reported mutations at the *SLC20A2* gene, which seems to be responsible for ~50% of the families with this disease. This gene encodes for an inorganic phosphate transporter (PiT-2), a transmembrane protein with varied expression in several tissues, that performs a housekeeping function in Pi homeostasis. Recently, PBC was also associated to another inorganic phosphate transporter coding gene, *XPR1*, which is the first inorganic phosphate exporter to be reported in metazoans. The other two PBC genes are *PDGFβ* and its receptor *PDGFRβ*, both involved in the maintenance of the blood-brain barrier.

With the intention to avoid redundancies and thereby make the reading experience of this thesis more fluid, the structure of the manuscript here presented was elaborated in order to integrate the doctorate candidate's published scientific production with the remaining results found during this work.

The bibliographic review was replaced by the author's published book chapter "An update on Primary Familial Brain Calcification" of *International Review of Neurobiology*'s special volume 110: "Metal Related Neurodegenerative Disease". The following section, "Material and Methods", presents the mostly used experimental procedures which were not described in later sections correspondent to a scientific article. Next in order, there are sections of the respective three published scientific articles and the section "General Results and Discussion", where results that have not already been published or submitted to publication are discussed, followed by a final "Conclusions" section.

## 1.1. PROBLEMATIZATION

It is estimated that approximately 1% of computed tomography (CT) scans present signals of calcium phosphate deposits in the basal ganglia region, including physiological calcifications consequential to ageing observed in older patients.

However, when found in individuals younger than 40 years old, basal ganglia calcifications are usually considered pathological and it is imperative to determine its cause. Usually the diagnosis is done by exclusion, which has been challenging because the neuroimaging finding is frequently associated with heterogeneous symptoms whose could be secondary to a number of conditions, such as infections, hormonal disorders and neurodegenerative syndromes.

When apparently no previous condition can be identified, the calcifications are considered idiopathic or primary. Since 2012, four genes have been associated with primary brain calcifications (PBCs), allowing faster and more accurate diagnosis by genetic screening and giving support to elucidate the molecular mechanisms of calcification formation. The analyses performed in this thesis can aid the diagnosis of patients not only by the screening of this four genes, but also by the possible identification of PBC new candidate genes. Moreover, a better comprehension of the genetic and molecular bases of PBC is fundamental for the future development of new treatments for this disease.

## 1.2. OBJECTIVES

### 1.2.1. General objective

To identify the genetic bases of PBC and to screen candidate genetic variants by sequencing analyses.

### 1.2.2. Specific objectives

- To screen by Sanger sequencing variants of the PBC-associated genes *SLC20A2*, *XPR1*, *PDGFB* and *PDGFRB* in genomic DNA samples of Brazilian patients with brain calcifications;
- To search by Sanger sequencing new candidate genes, mainly the other members of the platelet-derived growth factor family, *PDGFRA*, *PDGFA*, *PDGFC* and *PDGFD*, as well as *PCDH12*;
- To verify, by bioinformatic prediction tools, the biological consequences of variants found by sequencing;
- To validate the detected mutations which are rare or new and which have been predicted by bioinformatic as affecting gene function.

## 1.3. BIBLIOGRAPHIC REVIEW

Chapter: "An Update on Primary Familial Brain Calcification". In: *International Review of Neurobiology*, vol. 110 - Metal Related Neurodegenerative Disease, p. 349-371, 2013. doi: 10.1016/B978-0-12-410502-7.00015-6.

CHAPTER FOURTEEN

# An Update on Primary Familial Brain Calcification

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## Abstract

Patients with primary familial brain calcifications (PFBC) present bilateral calcifications, often affecting basal ganglia, thalamus, and cerebellum, inherited in an autosomal dominant pattern of segregation. Affected individuals display a wide variety of motor and cognitive impairments such as parkinsonism, dystonia, migraine, dementia, psychosis, and mood symptoms. Worldwide growth in the availability of neuroimaging procedures, combined with careful screening of patients and their relatives, has increased detection of PFBC. Recently, mutations in the *SLC20A2* gene coding for the inorganic phosphate transporter PiT2 were linked to PFBC, thereby implicating impaired phosphate transport as an underlying disease mechanism. To date, around 20 families of various ethnicities carry different mutations in *SLC20A2* correlate with ~40% of PFBC cases. More recently, two French families were recently reported with mutations in *PDGFRB*: c.1973 T > C, p.L658P and c.2959C > T, p.R987W, a class III tyrosine kinase receptor. Six other families were found with mutations in PDGFB, and, in general, mutations at the *PDGF pathway* add a new dimension to the physiopathology of PFBC so far explained by a disturbance in phosphate homeostasis with *SLC20A2*. The identification of *SLC20A2*, *PDGFRB*, and *PDGFB* provides a new avenue for potential treatments based on compounds such as bisphosphonates and those modulating the *PDGFB* pathway.

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## 1. INTRODUCTION

Ectopic calcifications are often damaging in soft tissues such as vessels, heart valves, lungs, kidney, and brain. This is often taken for granted as an age-dependent process, but recent data suggest that a myriad of molecules might modulate this process in an active fashion (Nitschke & Rutsch, 2012).

Brain calcification (BC) is a nonspecific neuropathology often associated with various chronic and acute brain disorders including Down's syndrome, Lewy body disease, Alzheimer's disease, Parkinson's disease, vascular dementia, brain tumors, and various endocrinologic conditions (Oliveira, 2011).

The pathology of BC is based upon calcium and other mineral deposits in blood vessel walls and tissue parenchyma causing neuronal death and gliosis. Calcification (mineralization) may be triggered by metabolic and inflammatory disorders, but it is also controlled by genetic predisposition. It has been suggested that deposits are not the source of the symptoms but rather a sign of progressive underlying tissue damage (Sobrido, Copolla, Oliveira, Hopfer, & Geschwind, 2013).

Vascular, as well as brain parenchymal, calcification is found in the basal ganglia and other brain areas including the cerebellum, thalamus, and brain stem. Patients presenting such calcifications display a wide variety of motor and cognitive impairments, such as parkinsonism, dystonia, dementia, psychosis, and mood symptoms. Some individuals may be symptom-free, despite extensive signs of calcification; however, studies measuring the total volume of calcification suggest that there are far more deposits in symptomatic relative to asymptomatic subjects. Indeed, this often leads to misdiagnosis (Manyam, 2005).

Despite the different etiologies, BCs often present a similar composition, that is, an amalgam of hydroxyapatite with a major content of calcium phosphate. Electron microscopy suggests that an initial *nidus* is calcified, with a progressive increase in volume and area affected. In some cases, the calcification is self-limited in terms of growth and expansion, and some authors suggest that this process starts with neuronal mitochondria (Maetzler et al., 2009). Calcium deposits found in both human brain and animal models of neurodegeneration have a crystalline structure composed of biological hydroxyapatites similar to those in peripheral tissues. On the other hand, nonatherosclerotic calcium deposits present in vascular dementia, Alzheimer's disease, Parkinson's disease, Lewy body disease, epilepsy, and astrocytoma are very similar, despite the pathology (Honda et al., 1994;

Khan & Hackett, 1986; Kim, 1995; Mahy, Prats, Riveros, Andre's, & Bernal, 1999; Rodríguez, Bernal, Andrés, Malpesa, & Mahy, 2000).

Several different imaging procedures can be used to detect BC, and even traditional head radiography can show anomalies associated with calcification through the skull in advanced cases. Computerized tomography (CT) is the most appropriate neuroimaging technique to detect BC with studies in different populations (controls, affected, young, and senior) showing that its prevalence rate ranges from 1% to 20%, in an age-dependant pattern (Forstl, Krumm, Eden, & Kohlmeyer, 1992; Yamada et al., 2012).

Sporadic cases are reported more often than ever, with the growing availability of neuroimaging exams, suggesting that this phenotype is more common than previously assumed (Oliveira & Steinberg, 2010). Baba, Broderick, Uitti, Hutton, and Wszolek (2005) revised the most frequent familial forms of BC, including conditions with calcium, phosphorus, and parathormone disturbances according to familial forms of isolated hypoparathyroidism, autoimmune polyglandular syndrome, and pseudohypoparathyroidism. However, there are also a number of conditions where there are no obvious metabolic or hormonal changes, that is, Aicardi–Goutieres syndrome, Cockayne syndrome, polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy, autosomal dominant chromosome instability syndrome, Krabbe disease, mitochondrial myopathy, encephalopathy, and lactic acidosis.

For diagnosis, a thorough analysis should be done for parathyroid hormone levels in serum as well as for calcium, and phosphorous (including an Ellsworth–Howard test to measure serum and urinary phosphorus after intravenous administration of parathyroid extract, testing for pseudohypoparathyroidism). Additional analysis of serum electrolytes, thyroid and growth hormone, sedimentation rate, renal and liver function tests, cortisol, ceruloplasmin levels, and viral markers should be performed (Oliveira, 2011).

Other examinations including karyotype, muscle biopsy, and mutation screens will be required in some rare conditions such as mitochondrial encephalomyopathy, stroke-like symptoms, lactic acidosis, or other genetic disorders such as Down's syndrome (Sobrido et al., 2013). Several of these conditions manifest themselves with calcifications in various sites, with additional metabolic disturbances. In these instances, the brain is affected not as a primarily organ but as a secondary target. Some authors have tried to find patterns of calcification by neuroimaging phenotypes and comparing different intracranial calcification disorders. For example, Livingston, Stivaros, van der Knaap, and Crow (2013) suggested a classification based on the following categories considering the most affected structures: probable destructive

mechanism; calcification associated with polymicrogyria; familial calcification with involvement of the globus pallidus, posterior limb of the internal capsule, genu of the corpus callosum, and deep white matter; and dominant familial calcification "Fahr's disease." However, the authors admit the limitations of such an approach as there are considerable variations in standard procedures and heterogeneous data analysis approaches for neuroimaging protocols. In addition, the authors recognize that this study is biased due to a set of scans sent to the coauthors for a diagnostic opinion that was nonblinded. The authors are currently seeking to improve confidence through the inclusion of additional data sets (more images of patients with and without known diagnosis). However, it is unlikely that a specific pattern could be found as we often see variations within families, even within the same generation. On the other hand, our group reported a small family with identical twins affected by BC and with a noteworthy, almost identical pattern of calcification, anatomical distribution, and predicted volume (Oliveira, Lima, & Zatz, 2009). Curiously, the brain appears to be prone to calcification, even in conditions where there is no associated cause (often referred as Fahr's disease "idiopathic"). This is why it is important to understand this condition in a model such as primary familial brain calcification (PFBC), where the first associated genes were recently identified (Oliveira, Oliveira, Lemos, & Oliveira, 2009).

## 2. PRIMARY FAMILIAL BRAIN CALCIFICATION

The term *primary familial brain calcification* was recently suggested by Sobrido et al. (2013), after considering the previous terms (Fahr's disease or familial idiopathic basal ganglia calcification) obsolete. Firstly, there are now clear genetic culprits, and secondly, calcifications can also appear in other brain regions. However, the basal ganglia still seems to be a more frequently affected brain region. Diagnosis of PFBC is based on clinical history, neuroimaging analysis, and complementary exams to test for biochemical abnormalities, infections, toxicity, and other causes (Ellie, Julien, & Ferrer, 1989; Manyam, 2005; Moskowitz, Winickoff, & Heinz, 1971).

Defining affected individuals is challenging due to heterogeneity in clinical presentation, age-dependent penetrance, and the fact that many asymptomatic individuals have positive CTs. Thus, we defined affected individuals as those with positive CTs as previously described (Oliveira et al., 2004; Sobrido et al., 2013; Sobrido & Geschwind, 2001). Biochemical investigation should be performed in at least one affected individual per family to rule

out abnormalities of calcium regulation and metabolic disorders such as pseudohypoparathyroidism that could underlie brain calcifications. Those with negative CTs who are over the age of 50 are defined as unaffected, whereas those at earlier ages are classified as unknown because of the age-dependent penetrance for calcium deposits (Geschwind, Loginov, & Stern, 1999).

In this chapter, we will focus on the recent genetic finds associated with PFBC.

## 3. INHERITANCE PATTERNS AND GENES LINKED TO PFBC

Most families reported with PFBC present an autosomal dominant pattern of segregation with only a few kindred reported with recessive inheritance. Several other families have been described more recently, and the list grows as neuroimaging procedures become more widely available, allowing screening of patient's relatives (Baba et al., 2005; Brodaty et al., 2002; Dai et al., 2010; Geschwind et al., 1999; Lhatoo, Perunovic, Love, Houlden, & Campbell, 2003; Oliveira et al., 2003, 2004; Volpato et al., 2009; Wszolek et al., 2006).

Recently, mutations in the *SLC20A2* gene coding for the inorganic phosphate transporter PiT2 were linked to PFBC, thereby implicating impaired phosphate transport as an underlying disease mechanism (Wang et al., 2012). *SLC20A2*, located on chromosome 8, was initially identified as a retrovirus receptor (Kavanaugh & Kabat, 1996). Expression of SLC20A2 occurs in several tissues including bone, vascular smooth muscle cells, parathyroid glands, kidney, intestine, and brain, performing a housekeeping function in inorganic phosphate ($P_i$) homeostasis (Bøttger & Pedersen, 2011; Villa-Bellosta et al., 2009; Villa-Bellosta & Sorribas, 2010).

Bøttger and Pedersen (2002) reported that p.E575K (a region containing a highly conserved glutamate residue) is critical for $P_i$ transport activity of PiT2. Intriguingly, an independent study later demonstrated this same region contained a number of mutations in PFBC patients (Wang et al., 2012). These same mutations were inserted into *Xenopus* oocytes, resulting in impaired inorganic phosphate uptake. This suggests that these mutations may be associated with haploinsufficiency rather than by encoding dominant-negative activities (Wang et al., 2012). To date, around 20 families of various ethnicities have been reported to possess different mutations in

*SLC20A2*, and this gene is associated with ~40% of PFBC cases (Hsu et al., 2013; Lemos, Oliveira, & Oliveira, 2013; Nicolas et al., 2013; Schottlaender et al., 2012; Zhang, Guo, & Wu, 2013).

Mutations in *SLC20A2* have been reported as missense, frameshift, deletion, nonsense, synonymous, and in splice site. These rare mutations have not yet been listed in databases of genetic variations such as Exome Variation Analyzer (EVA), dbSNP, 1000 Genomes, Exome Sequencing Project (ESP), or HapMap. *In silico* analysis using PolyPhen-2, SIFT, I-mutation, and Human Splicing Finder predicts most of these mutations to be deleterious (Table 15.1).

Hsu et al. (2013) examined a large cohort of PFBC families in 218 patients from 29 PFBC families with mutations accounting for 41% (or 12 out of 29 of the cases). Interestingly, nine out of the 14 mutations were predicted to introduce a stop codon, pointing also to haploinsufficiency as a mechanism (Hsu et al., 2013). Other mutations in *SLC20A2* were also seen in individuals from nuclear families or unrelated individuals (Lemos et al., 2013; Nicolas et al., 2013).

Importantly, Zhang et al. (2013) assessed the relative expression of *SLC20A2* transcripts by using qRT-PCR in PFBC patients. The deletion of c.510delA (within *SLC20A2*) produced a truncated PiT2 protein with a premature termination codon. This resulted in a reduction in *SLC20A2* mRNA expression (~30%) in tissues from affected individuals relative to biological controls (Zhang et al., 2013). Despite these interesting findings, a deeper understanding of the relationship between clinical expression, findings of calcifications, and genetic mutations in PFBC needs to be established. So far, no obvious genotype–phenotype correlation in study families with *SLC20A2* mutations has been observed. Phosphate imbalance has been associated with vascular calcification *in vivo* (Block, Hulbert-Shearon, Levin, & Port, 1998). In addition, culturing cells in the presence of increasing concentrations of $P_i$ leads to calcium deposition in a dose-dependent fashion (Giachelli, 2003).

The mechanism that underlies $P_i$-mediated plaque formation is relatively poorly understood. However, it has been reported that high $P_i$ levels activate the transcription factor cbfa1 leading to induction of osteogenic markers (Giachelli et al., 2001).

Curiously, mutations have also been screened in the *SLC20A1* gene, but, as yet, none have been confirmed. *SLC20A1* deletion in a transgenic mouse model is lethal during embryonic development (Beck et al., 2010),

Table 15.1 Descriptions of the mutations in *SLC20A2* and *PDGFRB* genes

| No. | cDNA | Amino acid NP_006740.1 | Mutation type | Authors | GenBank reference |
|---|---|---|---|---|---|
| 1 | c.1492G > A | p.G498R | Missense | Wang et al. (2012) | NM_006749 (*SLC20A2*) |
| 2 | c.1802C > G | p.S601Q | Missense | | |
| 3 | c.1802C > T | p.S601L | Missense | | |
| 4 | c.1409delC | p.P470LfsX37 | Frameshift | | |
| 5 | c.1723G > A | p.E575K | Missense | | |
| 6 | c.1784C > T | p.T595M | Missense | | |
| 7 | c.124_126delGTG | p.V42del | Deletion | | |
| 1 | c.1723G > A | p.E575K | Missense | Schottlaender et al. (2012) | NM_006749.4 (*SLC20A2*) |
| 1 | c.508delT | p.L170X | Nonsense | Hsu et al. (2013) | NM_006749.3 (*SLC20A2*) |
| 2 | c.514A > T | p.K172X | Nonsense | | |
| 3 | c.583_584delGT | p.V195LfsX61 | Frameshift | | |
| 4 | c.760C > T | p.R254X | Nonsense | | |
| 5 | c.1101C > G | p.P367P | Synonymous | | |
| 6 | c.1145G > A | p.R382Q | Missense | | |
| 7 | c.1506C > A | p.H502Q | Missense | | |
| 8 | c.1523 + 1G > A | p.G312VfsX8 | Splice site | | |

*Continued*

Table 15.1 Descriptions of the mutations in *SLC20A2* and *PDGFRB* genes—cont'd

| No. | cDNA | Amino acid NP_006740.1 | Mutation type | Authors | GenBank reference |
|---|---|---|---|---|---|
| 9 | c.1652G > A | p.W551X | Nonsense | | |
| 10 | c.1703C > T | p.P568L | Missense | | |
| 11 | c.1794 + 1G > A | p.S570RfsX30 | Splice site | | |
| 12 | c.1794 + 1G > C | p.S570RfsX30 | Splice site | | |
| 13 | c.1802C > T | p.S601L | Missense | | |
| 14 | c.1828_1831delTCCC | p.S610AfsX17 | Frameshift | | |
| 1 | c.1483G > A[a] | p.A495T | Missense | Lemos et al. (2013) | NM_006749.4 (*SLC20A2*) |
| 1 | c.551C > T | p.P184L | | Nicolas et al. (2013) | NM_006749.3 (*SLC20A2*) |
| 2 | c.431-1G > T | | Splice site | | |
| 1 | c.510delA | p.R172fsX19 | Frameshift | Zhang et al. (2013) | n.a.[b](*SLC20A2*) |
| 1 | c.1973 T > C | p.L658P | Missense | Nicolas et al. (2013) | NM_002609.3 |
| 2 | c.2959C > T | p.R987W | Missense | | (*PDGFRB*) |

[a]Annotation corrected from the position reported in Lemos et al. (2013).
[b]n.a.: Not available.

suggesting that mutations in *SLC20A1* might be too deleterious and incompatible with embryonic survival.

Recently, Nicolas et al. (2013) reported two French families with mutations in *PDGFRB*: c.1973 T > C, p.L658P and c.2959C > T, p.R987W (Nicolas et al., 2013). *PDGFRB* is a class III TK receptor, localized on chromosome 5. Mutations in *PDGFRB* add a new dimension to the pathophysiology of PFBC, so far explained by a disturbance in phosphate homeostasis. Both *PDGFRB* mutations cause amino acid substitutions in the intracellular domain of *PDGFRB*, one of them affecting a conserved residue in the kinase domain. Six other families were recently found with mutations in *PDGFB*, but the functional consequences of these mutations, however, are not currently understood (Keller et al., 2013). The *PDGFB* pathway is involved in many mechanisms including angiogenesis, pericyte survival, and blood–brain barrier (BBB) maintenance. Previous studies have demonstrated that angiogenesis might be crucial to the formation of calcifications and that both processes might act in an adjuvant pathway (Table 15.1).

As a critical regulator of the BBB, mutation of *PDGFRB* is key to homeostasis in the central nervous system (CNS) (Daneman, Zhou, Kebede, & Barres, 2010). The inability to regulate and control the cellular environment in the CNS might represent an important step in the understanding of PFBC. Further, leakage of fluid derived from plasma through the vascular wall and into the CNS may be a factor leading to tissue damage and resulting in progressive calcinosis. This mechanism has been considered for a long time, based on neuroimaging and metabolic studies in idiopathic and nonidiopathic causes of BCs (Baba et al., 2005). Indeed, BBB integrity has been implicated in several neurodegenerative conditions including amyotrophic lateral sclerosis (Garbuzova-Davis et al., 2007), Alzheimer's disease (Zipser et al., 2007), and Parkinson's disease (Kortekaas et al., 2005). However, whether BBB dysfunction is a causative factor in these cases is a matter of debate. In the case of PFBC, why loss of BBB integrity would lead to preferential calcification of specific brain regions, that is, basal ganglia and not others remains to be determined. However, certain regions of the brain are more susceptible to noxious stimuli, for example, CA1 pyramidal neurons of the hippocampus are less resilient to stroke (Smith, Auer, & Siesjö, 1984).

Intriguingly, Giachelli et al. (2001) reported that PDGF-BB, the preferential PDGFRB ligand, can modulate the maximum velocity of $P_i$ uptake by the phosphate transporter PiT1 (*SLC20A1* protein product). This finding provides a possible link between the two genes currently implicated in the pathophysiology of PFBC.

Other rare disorders with vascular calcification such as generalized arterial calcification of infancy, pseudoxanthoma elasticum, and calcification of joints and arteries are linked to mutations in *ENPP1*, *ABCC6*, and *NT5E*, respectively (Nitschke & Rutsch, 2012). These might also represent additional candidate genes for PFBC, especially if we consider that, so far, the two genes linked to PFBC correspond to ~50% of all families studied so far. The unveiling of PFBC neurogenetics might also help to understand other neuropsychiatric conditions with similar clinical outcome.

## 4. PENETRANCE AND BCs

The study of asymptomatic individuals with extensive brain calcification suggests that the concept of penetrance can be understood in relation to brain lesions (where a threshold for the manifestation of the mutation with formation of calcifications can be observed).

Despite extensive calcifications in some oligosymptomatic and mild cases, the apparent functionality of affected brain structures is remarkably efficient, suggesting an intrinsic resilience mechanism and also a threshold for triggering the symptoms. A previous study suggested that it is necessary to consider two levels of penetrance in PFBC: the penetrance for the calcification formations and the penetrance for the clinical manifestation (Oliveira, Lima, & Zatz, 2009). Curiously, a resilience mechanism modulated by the cerebellum has been recently considered in dystonia. Neurogenetics is a specialty full of such examples, and the advent of imaging studies in association with genetic testing in familial dystonia allowed visualization of significant changes in the brains of asymptomatic carriers of mutations in the *DYT1* gene. The issue of differential levels of penetrance has already been addressed in other movement disorders such as primary torsion disease (PTD) and Huntington disease (HD) (Argyelan et al., 2009; Eidelberg, 2009; Kempton et al., 2009).

In our previous linkage study, we analyzed the ratio between symptomatic and asymptomatic individuals among patients with familial calcifications in the basal ganglia, showing that only 28 patients were clinically affected out of 47 patients with calcifications (Oliveira et al., 2004). This finding reinforces the surprising resilience to this type of brain injury. While both HD and PTD are characterized by autosomal dominant inheritance, they also present with variable penetrance. Compared with the HD carrier state, only a minority of mutated dystonia gene carriers ever develops clinical symptoms. Abnormal metabolic findings in the brains of asymptomatic

carriers show that our definition of genetic penetrance may be flawed when dealing with hereditable movement disorders with probable neuroimaging endophenotypes (Argyelan et al., 2009; Eidelberg, 2009).

The fact that such extensive calcified regions may remain asymptomatic over several decades reveals a singular mechanism of behavioral compensation against progressive calcinosis, suggesting continuous resilience and degeneracy of multiple neural systems (Noppeney, Friston, & Price, 2004).

The study of new families will be crucial to confirm current reports, but the discovery of actual genes responsible for PFBC will be the ultimate finding to establish the molecular basis of such intriguing phenotypes.

## 5. PENDING CANDIDATE LOCI AND OTHER RISK FACTORS

Previously, several candidate genetic regions have been suggested as being associated with the PFBC phenotype. However, at least one of them, such as the IBGC1 locus on chromosome 14, was recently revised and linked to mutations in *SLC20A2* (Hsu et al., 2013; Wang et al., 2012).

The first locus linked to this condition (IBGC1) was localized on the long arm of chromosome 14 (Geschwind et al., 1999). The first screening for possible mutations identified a heterozygous variation (rs36060072) at the *MGEA6/CTAGE5* gene, potentially pathogenic, present in all affected individuals of a large American pedigree and absent in controls (Oliveira et al., 2007). This missense substitution at exon 20 leads to proline to alanine substitution at position 521 (P521A). Exon 20 of the *MGEA6* gene is commonly spliced, generating the isoform MGEA 11, also expressed in the brain (Usener et al., 2003). Considering that the population frequency of a given variation is an indirect indicator of potential pathogenicity, we screened a control population and found two samples heterozygous for P521A among 174, bringing the minor allelic frequency (MAF) of this single nucleotide polymorphism (SNP) to 0.0058.

We recently screened 200 chromosomes in a random control set of Brazilian samples and in two nuclear families, comparing with our previous analysis in a US population. Our genetic screen found no P521A carriers, and pooling this data together with previous studies in the United States, we now have a MAF of 0.0036 (Lemos, Oliveira, Zatz, & Oliveira, 2011). The exon 20 SNP should be considered a rare variation based on the study of Freudenberg-Hua et al. (2003) who analyzed 65 candidate genes for CNS disorders and concluded that rare SNPs have MAF $<0.05$.

Computer-based modeling is often used to simulate pathogenicity of genetic mutations. However, the lack of protein data, including crystallography and structural modeling in the case of *MGEA6*, limits the strength of such simulations

Proline residues play an important role in the structure and function of various proteins. The insertion of an alanine (a nonpolar side-chain amino acid) is likely to have significant implications to the tertiary and quaternary structure of this protein (Macias et al., 1996; Macias, Wiesner, & Sudol, 2002; Sansom & Weinstein, 2000). In addition, we performed analyses through bioinformatics programs to predict the pathogenicity of such variations, which provided conflicting findings (Lemos et al., 2011).

Several aspects make *MGEA6* a great candidate for PFBC. *MGEA6* is a coil-coiled protein with a proline-rich region expressed in several tissues including brain. This type of proline-rich "signature" is related to regulatory pathways, through interaction with domains of other proteins that have affinity for sequences rich in prolines, namely, the Src homology 3 (SH3) and tryptophan-rich domains (WW). These are involved in a wide variety of cellular activities such as growth, cell cycle, transcription, synaptic signaling, and cell motility (Comtesse et al., 2002; Sudol et al., 2001; Zarrinpar et al., 2003). *MGEA6* is also highly expressed in meningioma, the most common benign brain tumor that often presents calcifications visible in neuroimaging studies, especially CTs (Comtesse et al., 2002; Comtesse, Reus, & Meese, 2001; Usener et al., 2003). Another interesting molecular overlap between meningiomas and bone-related proteins is seen in a study by Hirota et al. (1995). Hirota et al. (1995) suggested a possible involvement of osteopontin in the development of psammoma bodies, which are microscopic, calcified, extracellular bodies commonly found in meningioma.

Previous preliminary linkage studies performed by our group have highlighted other candidate regions. A 10cM genome-wide scan was performed in four families with a total of 47 subjects using an ABI MD 10 marker panel. An additional family with 14 more subjects is still under analysis.

The largest single-point LOD score was observed on chromosome 7 with a LOD score of 3.023 at marker D7S519 in the FB2 family (10 affected). The minimal interval defined by haplotype analysis is between the markers D7S484 and D7S506, spanning about 20cM. Three smaller families, with a total of 16 affected, had a maximum multipoint LOD score of 3.487 on chromosome 9 at a position roughly midway between D9S157 and D9S171. Haplotype analysis shows that these families share a common

region around 16cM between the markers D9S171 and D9S1817 (Oliveira et al., 2003). These results reinforce preliminary studies demonstrating genetic heterogeneity in PFBC and report two potential new candidate regions, one of them shared by three families. Additional fine-mapping analysis, SNP genotyping, and exome analysis will confirm further mutations.

Comparing the regions from three chromosomes linked by our group to PFBC (7, 9, and 14), we realized that some genes could be grouped based on a similar biochemical role. Linkage studies by Wszolek et al. (2006), in a large kindred with autosomal dominant dystonia and brain calcification, listed different regions of chromosomes 8 and 9 as PFBC-associated loci.

Additionally, Volpato et al. (2009) reported loci on chromosome 2, identifying several genes related to calcium metabolism located in 2q37, such as *INPP5* and *EFHD1* in a South Tyrolean family. Curiously, two families with Fried syndrome (X-linked mental retardation with hydrocephalus and calcifications in basal ganglia) have been reported in France and Scotland and bearing *AP1S2* mutations (Saillour et al., 2007).

The continuing research suggests more PFBC gene candidates might be located on chromosomes 2, 7, 8, and 9 demonstrating the genetic heterogeneity of this disease (Oliveira & Lemos, 2009; Oliveira, Lima, & Zatz, 2009; Oliveira et al., 2007; Volpato et al., 2009).

With the growing report of incidental findings of patients with PFBC (Oliveira & Steinberg, 2010), there is also a high chance that more families will be reported in the near future and with new mutations at *SLC20A2*, *PDGFRB*, *PDGFB*, or in other genes.

Recently, Da Silva, Pereira, and Oliveira (2013) described the gene expression pattern across the whole brain for SLC20A2 using the Allen Institute Human Brain Atlas database. Microarray analysis provided evidence that the neuroanatomical pattern of expression for this gene is highest in regions most commonly affected in PFBC. The following genes demonstrated high correlation with *SLC20A2* expression: *PCDH12*, *RHOBTB2*, *FLJ43860*, *VAMP1*, *EPB41L3*, *AGPAT9*, *NEFH*, *PVALB*, *CDR2L*, *EMC10*, and *SCN1B*. On the other hand, the following genes (anti-correlated) presented an "inverted" pattern of expression compared with *SLC20A2*: *FAM171B*, *GABRB1*, *CDH4*, *GABRA2*, *PNCK*, *MYH7*, *RBP4*, *SCN3B*, *C1orf173*, *ACTN1*, *MMD*, *LPPR3*, *C1orf194*, *FRRS1L*, *PKIA*, and *LMO3*. These genes likely point to a molecular network with potential to explain the relatively localized neuroanatomical distribution of calcifications in PFBC.

## 6. MODELING BCs

The most common animal models for BCs include knockouts, chemically induced calcification, or aged animals without any genetic or chemically interventions. The major challenge is to find a model that can actually present the calcifications spontaneously without an external triggering agent.

Previous reports of basal ganglia calcification in aged animals show cerebral mineralization in horses, cattle, monkeys, and aged dogs, with cognitive deficits together with histological alterations resembling those of Alzheimer's disease (Gavier-Widen, Wells, Simmons, Wilesmith, & Ryan, 2001; Petegnief et al., 1999; Ramonet et al., 2002; Towfighi, Yager, Housman, & Vannucci, 1996; Yanai et al., 1996, 1994; Yasui et al., 1997; Yasui, Yano, Tsuda, & Ota, 1991).

Calcium precipitation is part of the ongoing degenerative process in rodent brains and can be induced by microinjection of glutamate agonists such as ibotenic acid (IBO) or quisqualate (QUIS). Calcinosis is mainly composed of calcium and phosphorous amalgamate in a hydroxyapatite like composition (Bernal, Saura, Ojuel, & Mahy, 2000; Rodríguez et al., 2000; Rodríguez, Pugliese, & Mahy, 1999, 2009; Rodríguez, Robledo, Andrade, & Mahy, 2005; Saura, Boatell, Bendahan, & Mahy, 1995). As a result of calcification, cytoplasmic calcium ions precipitate as solid, spherical concretions that grow progressively in size and number (Mahy et al., 1999). Similarly, hydroxyaluminosilicates have also been observed in Alzheimer's disease and Parkinson's disease (Birchall, 1992; Dowe, Heitzman, & Larkin, 1992; Dusart, Marty, & Peschanski, 1991; Ellie et al., 1989; Hohmann, Wenk, Lowenstein, Brown, & Coyle, 1987; Mahy et al., 1999). There is a marked tendency of large deposits to develop into a spherical shape, and the presence of templates over which supercritical nuclei can be formed is also an important contributor to this mechanism. However, pathological analysis using electron microscopy shows that such aggregates undergo structural modifications over time, suggesting also that the aggregation *per se* might not be the main cause but rather a result of the neuronal damage process.

Conversely, other studies support the theory that such aggregates might have a protective effect, avoiding additional free $Ca^{2+}$ (Maetzler et al., 2009). After microinjection of glutamate agonists, extensive analysis demonstrated a limited neuronal loss and a normal morphology of oligodendrocytes in rats, with good preservation of cytoplasm and processes. Reactive

astrocytes and neurons frequently presented cytoplasmic, needle-shaped calcium deposits on a matrix close to cellular organelles, such as mitochondria, microtubules, or vesicles. Large extracellular deposits were surrounded by reactive microglia in a phagocytic-like process. Their formation does not depend on the glutamate receptor subtype initially stimulated. However, their size, number and distribution vary not only by excitotoxin but also by the location within the CNS. For example, the development of calcium deposits over time in rats with lesions induced in basal ganglia was more pronounced in lesions induced by IBO than with QUIS (Mahy et al., 1995, 1999).

A study with transgenic mice expressing the calcium binding protein parvalbumin in neurons showed that excitotoxicity with IBO accelerated the calcification process. It is also demonstrated that mitochondria may be the initial structure involved in triggering calcium deposits, leading to the hypothesis that these processes may be causative in the normally aging brain and in neurodegeneration (Maetzler et al., 2009).

The intracellular mucopolysaccharides shown in human and rodent calcification play an important role as modulators for mineralization (Adachi & Volk, 1968; Beall, Patten, Mallette, & Jankovic, 1989). It has been postulated that hydroxyapatite formation with further reductions in free $Ca^{2+}$ would take place as an alternative homeostatic step to reduce excitotoxicity (Ramonet et al., 2002).

Maetzler et al. (2010) studied knockout osteopontin mice and demonstrated an exacerbated co-occurrence of progressive secondary neurodegeneration and microcalcification induced by ibotenate, another glutamate analog. The authors suggested that osteopontin could have a protective role in this process.

Other alternative models were investigated by Lammie et al. (2005) who characterized basal ganglia calcification in BB/E rats with diabetes. Alternatively, thalamic calcification in vitamin D receptor knockout mice was detected and represents another potential target; however, patients with PFBC, by definition, show no vitamin D disturbances (Kalueff et al., 2006).

More recently, Jensen et al. (2013) showed that homozygous mice for the knockout cassette, L1L2-PGK-P, inserted after exon 2 and flanking exon 3 at the Slc20a2 gene, present calcifications in the thalamus, basal ganglia, and other brain regions, compatible with PFBC, demonstrating that reduced PiT2 expression causes BCs. This is the first Slc20a2 knockout reported as a model for loss-of-function mutations, associated with BCs.

More recently, Keller et al. (2013) reported a hypomorphic knockout mice for the PDGFB gene, presenting BCs in a similar pattern compared to humans, reinforcing the involvement of the BBB in such phenotype. Developing additional knockout mice for other mutations in *SLC20A2, PDGFRB, and PDGFB* is a major priority for understanding PFBC physiopathology and for testing new drugs.

## 7. NEW THERAPIES BASED ON NEW GENES

The report of three new genes associated with PFBC might help to enlighten the current guidelines for medical treatment. So far, there is no specific treatment for familial BC. The huge clinical heterogeneity shows that the symptoms are likely too variable to be treated by a single agent. Currently, treatment might be managed based on the main symptoms, and there are case reports of patients treated with various medications such anticonvulsants, mood stabilizers, antipsychotics, antiparkinsonism, anti-incontinence, analgesics, antidepressants, and benzodiazepines. The prognosis is also variable with some reports stating cases where the symptoms were transient after treatment. Yoshikawa et al. (2003) reported a pediatric case in which a 12-year-old girl with substantial calcinosis presented transient motor and mood symptoms and recovered completely after symptomatic treatment. A CT screening of other siblings showed asymptomatic subjects with calcinosis. However, most patients show a progressive deterioration, despite continuous treatment.

Reports of mutations in the inorganic phosphate transporter (*SLC20A2*) associated with PFBC support the concept of designing treatments according to a primary cause and not only the major symptoms. However, the most intuitive treatments, based on the possible blockage of calcium or phosphate transport, have limited descriptions, and so far, no consistent clinical trial was specifically oriented to treat this rare condition. Calcium channel blockers such as nimodipine have failed as a treatment according to anecdotal reports (Manyam, 2005).

In very few cases, bisphosphonates (BP) such as etidronate disodium had positive effects in reducing symptoms but failed to reduce deposits according to CT imagery. Following treatment of patients presenting parkinsonism, ataxia, spasticity, and dystonia, there were improvements in speech and gait, without improvements in spasticity, dystonia, ataxia, and calcifications (Loeb, 1998). In addition, two cases including: (i) An 8-year-old boy with headaches and seizures treated with etidronate disodium had alleviated

symptoms but with no apparent reduction in calcifications, and (ii) 45 patients were treated with the same medication to control seizures, and psychotic symptoms lead to an improvement of symptoms but no apparent loss of calcified deposits as imaged by CT (Loeb, Sohrab, Huq, & Fuerst, 2006). The authors proposed that neurological symptoms associated with BCs can be reduced with etidronate disodium, which is known to inhibit the microcrystal growth of hydroxyapatite. BP are more commonly used for the prevention and treatment of postmenopausal osteoporosis, bone metastases, Paget's disease, and prevention and treatment of glucocorticoid-induced osteoporosis. Other osteoporosis-related conditions such as immobility are also currently treated with this drug (Räkel, Boucher, & Ste-Marie, 2011). Most BP show affinity for mineralized bone, binding to hydroxyapatite, with preferential localization to sites of intensive bone remodeling helping to reduce resorption mediated by osteoclasts. Independent studies confirm that the synthesis of farnesyl pyrophosphate, an enzyme involved in lipid biosynthesis, is the main target for various BP. BP rapidly decrease the rate of bone turnover through reduction of bone resorption and osteogenesis. There was no progressive reduction of bone turnover markers with repeated annual administration, in the case of Aclasta®, and age does not seem to influence this effect (Räkel et al., 2011).

Prospective and longitudinal studies that directly relate neurological symptoms to the formation of macroscopic calcifications as well as large-scale clinical trials with BP to both prevent and treat cerebral calcifications could be an effective approach to treat patients with brain calcinosis.

The identification of *SLC20A2*, *PDGFRB*, and *PDGFB* provides a new avenue for potential treatments based on the use of BP and drugs modulating the *PDGFRB* pathway such as imatinib. Imatinib is a tyrosine kinase inhibitor currently approved for the treatment of various cancers in the United States, and PDGFRB represents an interesting target for therapy as it is a kinase receptor activating many downstream pathways.

There are still several questions regarding this unusual neuropsychiatric condition; however, the reports of new genes as well as increased availability of detection methods bring a promising perspective for patients, clinicians, and basic scientists. Hopefully, this will improve our basic knowledge concerning ectopic calcification mechanisms and eventually benefit other conditions that share molecular and clinical overlapping with PFBC.

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## 1.4. MATERIAL AND METHODS

### 1.4.1. Patients and Samples

Probands previously investigated with full blood and urine screening as well as with bilateral brain calcifications in the region of the basal ganglia observed by neuroimaging analysis were selected for this study. Each patient signed a informed consent and had peripherial blood samples colected. This work was approved by the UFPE Ethics Committee (CAAE-0296.0.172.000-08 and CAAE - 09475912.8.00005208).

### 1.4.2. DNA Extraction

Genomic DNA was extracted by salting-out protocol according to Miller et al. (1988), with modifications described below, or with Wizard® Genomic DNA Purification Kit (Promega) or with FlexiGene DNA Kit (Qiagen) following manufactors' instructions. The DNA concentration and purity was obtained by measuring the absorbance at 260 nm with a NanoDrop® (Thermo Scientific) spectrophotometer. DNA aliquotes were diluted to 30 ng/uL or 100 ng/uL.

1.4.2.1. Salting-out DNA extraction

a) 10mL of anticoagulated blood (colected in EDTA tubes) were mixed by inversion until homogenization and transferred to a 50 polypropylene tube.
b) Add 35mL of 1 x Blood Lysis Buffer (see preparation of stock solutions below), mix vigourosly and then rest in ice for 30 min to lysate red blood cells.
c) Centrifuge at 1.800 rpm for 15 min and discard supernatant. Add 10mL of 1 x Blood Lysis Buffer, centrifuge at 1.800 rpm for 5 min and discard the supernatant again. You will obtain a pellet of white blood cells.
d) Resuspend the leukocytes pellet in 3 mL of 1 x Nuclei Lysis Buffer and mix well.
e) Add 100 µL of protease K (20 mg/mL) and 300 µL of 10% SDS. Mix carefully and then incubate at 65ºC for 1 hour.
f) Add 1 mL of 6M NaCl and vortex for15 seconds.
g) Centrifuge for 2.500 rpm for 20 min.

h) Transfer supernatant to a new 50 mL polypropylene tube and precipitate the DNA adding 2x volume of 100% ethanol.
i) Collect precipitated DNA cloud with a glass capillar or a plastic micropipet tip. Transfer the DNA to a 1,5 mL microtube and wash it with 1 mL of 70% ethanol.
j) Centrifuge at 12.000 rpm for 5 min, discard the supernatant and let the DNA pellet dry at room temperature for 30.
k) Resuspend the DNA in 200-500 µL of TE buffer and incubate at 65 ºC for 30 min to eliminate DNase contamination. Stock at -20 ºC.
l) Stock Solutions:
   i. 10 x Blood Lysis Buffer
      1550 mM NH4Cl
      100 mM KHCO3
      10 mM EDTA (pH 7.4)

   ii. 10 x Nuclei Lysis Buffer
      100 mM Tris-HCl (pH 8.0)
      4 M NaCl
      20 mM EDTA (pH 8.2)
   iii. TE Buffer
      10 mM Tris-HCl (pH 8.0)
      1 mM EDTA

### 1.4.3. Sanger Sequencing

The screening of genetic variants was obtained by PCR amplification followed by Sanger sequencing of the coding sequence of each selected gene, using cycle conditions previously described by Wang et al. (2012).

The four associated genes *SLC20A2* (Wang et al., 2012), *PDGFRB* (Nicolas et al., 2013), *PDGFB* (Keller et al., 2013) and *XPR1* (Legati et al., 2015) were analyzed, as well as candidate genes from the PDGF family – according to Hoch et al. (2003): *PDGFA*, *PDGFRA*, *PDGFC* and *PDGFD* – and *PCDH12*. For the first group, primer pair sequences were available in the literature cited above. For the second group, primer pairs were designed with Primer3 online software. Generally, each primer pair gives an amplicon correspondent to a coding exon.

Every reference sequence variation found by sequencing was annotated in our internal database and then searched in online databases dbSNP, Exome Variant Server, and Ensembl, or in Alamut Visual (Interactive Biosoftware) software when available. Known mutations generally had information regarding to minor allele frequency (MAF) available. In this case, only rare variants (MAF <2%) were considered as candidates.

In order to verify possible alterations in gene function, the variants were run by online bioinformatic prediction tools MutationTaster2, PolyPhen2 and SIFT.

### 1.4.4. Quantitative Multiplex PCR of Short fluorecent Fragments - QMPSF

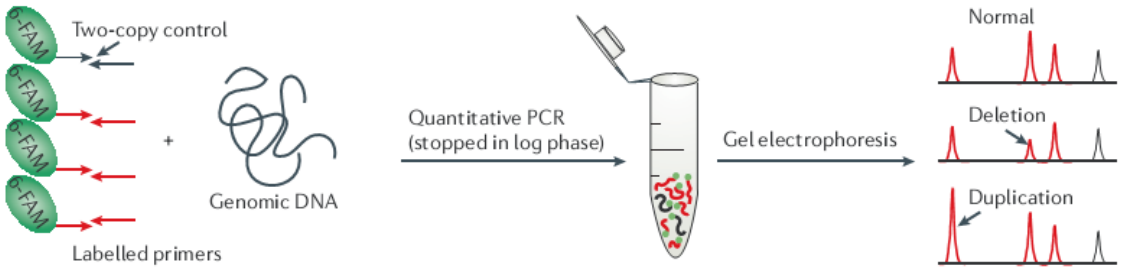
The design and optimization of the *SLC20A2* QMPSF reaction was performed by Joana FERREIRA and by Stéphanie DAVID (Inserm U1079, Roeuen, France). Then, Stéphanie DAVID screened the French PBC cohort (see section 9 for more details), while Joana FERREIRA was responsible for the screening of the Brazilian patients with brain calcifications.

A total of 14 primer pairs were designed for *SLC20A2* QMPSF reaction (see section 9 for primer sequences). In order to avoid unwanted primer interactions, a careful primer design is fundamental for QMPSF. PrimerPremier (Biosoft) was utilized to design primer pairs for each *SLC20A2* exon, as well as for an exon of *HMBS* control gene. The following parameters were regarded: amplicon's size should range between 100-300 bp with a difference of at least 10 bp among each one, and primers' annealing temperature should be 58-62 ºC (approximately 60 ºC). PrimerPremier tool "Multiplex PCR" was utilized to predict primer interactions, as the formation of homodimers, heterodimers and hairpins. Only primers with entalpy $\Delta G > -10$ kcal/mol were selected. Primer sequences were checked for absence of known variants with both SNPcheck and Alamut Visual (Interactive Biosoft) softwares. The sequences were also analyzed by BLASTN using "Human Genome (GRCh38.p2 reference assembly top-level)" and "megablast (highly similar sequences" parameters; only primers with e-value <0.2 were accepted. All foward primers were labeled with FAM fluorescent dye.

The QMPSF reaction was optimized not only by a temperature gradient to experimentally confirm that 60 ºC was the best annealing temperature, as well as verifying which were the exponential/quantitative phase cycles. After QMPSF, the ROX-labeled molecular size marker (GS-400 HD) and formamide were added to the reaction of each DNA sample, which were denatured by 2,5 min at 95 ºC. The reaction

plate was then read by an ABI 3100 instrument under fragment analysis parameters (GeneScan). The electropherogram obtained for each patient is superimposed to the electropherogram of a control DNA, and the peak of *HMBS* control gene is aligned between patient and control DNA. For analysis details see Charbonnier et al. (2000).

Figure 1. QMPSF schematic representation. Multiple DNA targets are amplified by FAM-labeled primer pairs used under quantitative conditions.




Source: Adapted from Feuk et al. (2006).

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# First Report of a De Novo Mutation at *SLC20A2* in a Patient with Brain Calcification

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**Abstract** Primary familial brain calcification (PFBC) is identified by mineralization of the basal ganglia and other brain regions in the absence of known causes. The condition is often inherited in an autosomal dominant pattern and can manifest itself clinically with neuropsychiatric symptoms such as Parkinsonism, headaches, psychosis, and mood swings. Mutations in the *SLC20A2* gene account for ~40 % of inherited cases, and this gene encodes an inorganic phosphate transporter (PiT-2), a transmembrane protein associated with Pi homeostasis. The p.Y386X mutation in *SLC20A2* was identified in a patient who presented migraines, brain calcification, and mild but chronic hypovitaminosis D. *SLC20A2* c.1158C>G single-nucleotide heterozygous mutation results in a premature stop codon and a putative truncated protein of 385 amino acids. Proband parents do not present the mutation, which is also not present in major public SNP databases, suggesting a de novo sporadic trait. This study describes for the first time a de novo *SLC20A2* mutation in a PFBC patient with migraine and mild hypovitaminosis D. This data further reinforces the pathogenic role of *SLC20A2* mutations as causal factors in PFBC physiopathology.

**Keywords** *SLC20A2* · Brain calcification · Vitamin D · Inorganic phosphate · De novo mutation

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## Introduction

Calcification of the brain can occur secondarily to other conditions such as infections (e.g., neurocysticercosis) and hormonal and biochemical imbalances. However, the terms "idiopathic basal ganglia calcification" or "Fahr's disease" were often used as synonyms to refer to cases without an apparent etiology. More recently, a new nomenclature suggests "primary familial brain calcification" (PFBC) as more appropriate for diagnostic purposes (Sobrido et al. 2013).

Common clinical manifestations include Parkinsonism, psychosis, dementia, and headaches, with an autosomal dominant pattern of inheritance for calcifications in the basal ganglia, thalamus, and dentate nuclei. In 2012, the first mutations associated with PFBC were reported in *SLC20A2*

(chromosome 8) encoding the ubiquitously expressed inorganic phosphate transporter PiT-2. Currently, almost 40 *SLC20A2* mutations have been associated with PFBC (Bøttger and Pedersen 2002;Wang et al. 2012; Nicolas et al. 2014b).

More recently, mutations in platelet-derived growth factor (*PDGFB*) and its receptor *PDGFRB* genes have been found in multiple PFBC patients. This data suggests that several pathways might lead to a similar phenotype (Keller et al. 2013; Nicolas et al. 2013).

Here, we report the discovery of the first de novo *SLC20A2* mutation in a Brazilian patient with brain calcification found during the investigation of a chronic migraine.

## Methods

### Subjects and Samples

An index case presenting chronic headaches was initially investigated with full blood and urine screening as well as neuroimaging analysis. Screening was also performed in three of the patient's relatives. CT scans were available only for subjects I-2, II-1, and II-2. Peripheral blood samples were collected from four subjects: index case and three relatives (Fig. 1a). A skin biopsy was also performed on the proband for DNA and RNA analysis. This project was approved by the UFPE Ethics Committee with signed informed consent forms from each subject.

### DNA Sequencing

Genomic DNA from blood and skin was isolated by salting-out protocol. PCR amplicons for *SLC20A2* exons were generated from blood DNA and sequenced by the Sanger method, using primers and cycle conditions described previously (Wang et al. 2012). Exon variants were analyzed using CLC Main Workbench software (Qiagen, USA). The identified mutation was verified by PCR and sequencing with five replicates in the proband and two for each relative. To confirm the biological relationship between the parents and proband, DNA was amplified and short tandem repeat (STR) analysis was performed with "Identifiler® Plus" (Life Technology, USA) and "PowerPlex® 16 HS" systems (Promega, USA) using 18 loci.

### RNA Analysis

Quantitative real-time PCR (qRT-PCR) was performed to quantify *SLC20A2* messenger RNA (mRNA) expression in samples using the ΔΔCT method. Total RNA was isolated from blood using PAXgene Blood RNA System (Qiagen, #762174, Switzerland). Reverse transcription was performed for each sample using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific Bio, #K1632, USA) in a 20-μL reaction containing 500 ng of total RNA. Real-time reactions were as follows: 1 μL complementary DNA (cDNA), 3.5 μL $H_2O$, and 5 μL TaqMan® Gene Expression Master Mix (Applied Biosystems, #4369510, Foster City, CA) with 0.5 μL of 20× TaqMan Gene Expression Assay (Hs_00198840_m1; Applied

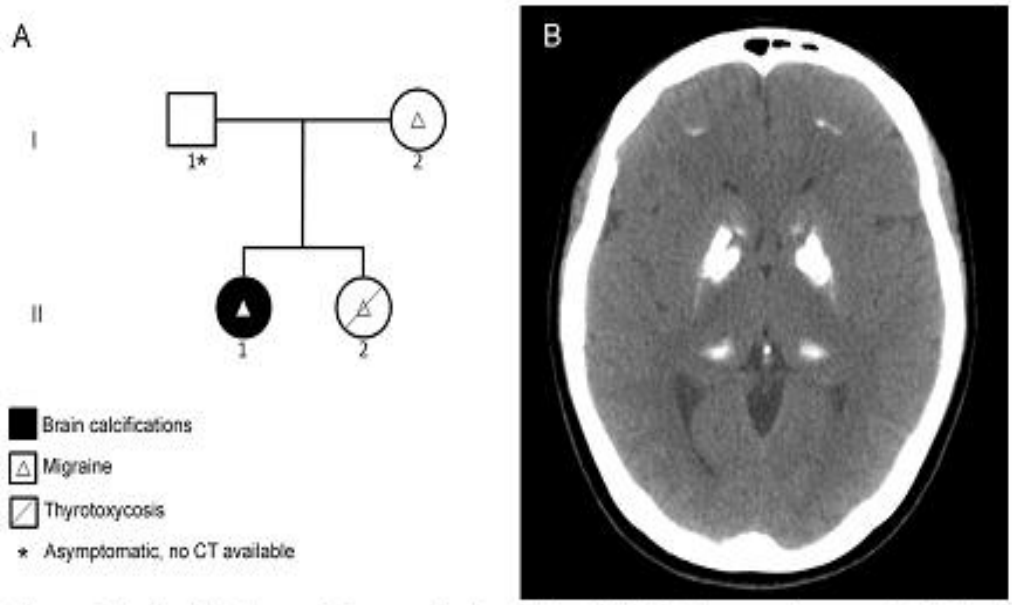


**Fig. 1** Pedigree analysis and positive CT scan of index case with migraine and hypovitaminosis D. **a** Pedigree of the index case indicating the subjects with brain calcifications (only II-1) and those with migraine (*I-2*, *II-1*, and *II-2*). II-2 also presents thyrotoxycosis. **b** Horizontal CT scan slice showing spots of calcification in the basal ganglia for the proband

Biosystems) or 20× TaqMan® Endogenous Control (Hs_02758991_g1; Applied Biosystems) in a 10-μL reaction. Real-time PCR reactions were run in triplicate and run using a 7500 Fast Real-Time PCR system (Applied Biosystems).

PCR-amplified cDNA fragments were then sequenced with automatic Sanger sequencing using the primers as follows: forward (5′-TCT GCT GCA CAA AAT CCA CA-3′) and reverse (5′-ACG GGT GTA GCT GCT TCT TG-3′).

## Results

### Clinical Features of Patient with *SLC20A2* De Novo Mutation

A 33-year-old female (mother of two children, employed as a real estate manager) was under investigation for chronic migraine, with mild functional impairment that had progressed recently. The chronic migraine had characteristically a pulsating pattern, was sensitive to light, and sometimes during over 24 h. This initially took her to an ER, and a neurological follow-up detected the calcifications. Various medications were used to alleviate this intermittent symptom. The patient also referred to regular menses not linked to a regular menstrual cycle. No neurological deficits were observed on physical examination. A CT scan revealed bilateral calcifications in the *globus pallidus* and pulvinar region of the thalamus and less intensely in the dentate nucleus. Sparse calcifications also presented in the white matter of the cerebellum and frontal subcortical regions (Fig. 1b). Hemoglobin, ceruloplasmin, and serological testing for toxoplasmosis, CMV, HIV, and hepatorenal biochemical markers were normal. Adenohypophyseal hormones such as prolactin, GH, TSH, FSH, and LH, together with cortisol, glucose, and lipids, were all within normal levels. Biochemical and endocrinological tests revealed only mild hypovitaminosis D (16 nmol/L of 25 hydroxyvitamin D [25(OH)D]). A supplementation was prescribed, but no change was detected at serum level follow-up (15 nmol/L of 25(OH)D). The proband reported a familial history of dementia (mother and maternal aunt) and thyrotoxicosis (Graves' disease) in a younger sister. Neuroimaging analysis of both mother and sister showed no calcification (data not shown).

### Screening and Validation of a De Novo *SLC20A2* Mutation

We identified the new mutation p.Y386X in exon 10 (previously exon 8) of the *SLC20A2* gene (NM_006749.4:c.1158C>G; NP_006740.1), which results in a premature stop codon and a putative truncated protein with 385 amino acids instead of 652 (Fig. 2). This mutation was not observed in the online databases *EVS* and *1000 Genomes* or in 55 controls from the *Inserm/Ruen exome* databases. Three relatives of the patient (two generations) were screened for the mutation, all of which showed negative (Figs. 1a and 2c, d). The biological relationship between the proband and parents was confirmed by multiplex STR analyses (data not shown). Since somatic mosaicism is common in subjects with rare disorders and de novo mutations, we screened another tissue from a different embryonic origin, which also contained the mutation. We also analyzed mRNA expression in RNA extracted from peripheral blood of the proband by RT-qPCR. *SLC20A2* expression was decreased by ~10 % (data not shown) in the proband. cDNA sequence reads show a reduced peak

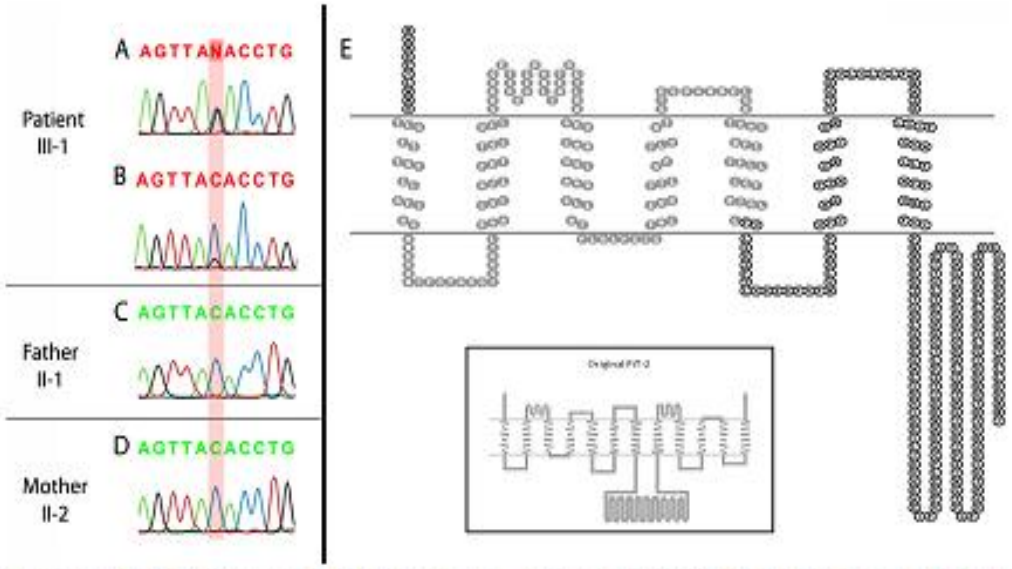


Fig. 2 De novo mutation in *SLC20A2* gene. **a**, **b** Electropherograms showing a *SLC20A2* de novo mutation in the patient (NM_006749.4:c.1158C>G), both in genomic DNA (**a**) and cDNA (**b**). This variant is absent in parents (**c**, **d**). Schematic diagram of wild-type and mutant *SLC20A2* proteins highlighting a shorter putative PiT-2 protein due to a stop codon at the 386 amino acid residue (NP_006740.1: p.Y386X) (**e**). Both models were generated by TOPO2 online software (http://www.sacs.ucsf.edu/TOPO2/)

correspondent to the mutant allele (Fig. 2b), comparatively with the genomic DNA (Fig. 2a).

## Discussion

This is the first report of a de novo mutation in the *SLC20A2* gene, reinforcing the causative role of pathogenic variations of this gene in PFBC.

Curiously, this patient also presents mild but chronic hypovitaminosis D. In 2012, the patient was diagnosed with hypovitaminosis D, and despite prescription of supplements, there was no change (parathyroid hormone was within the normal range in both instances). Chronic hypovitaminosis D is influenced by several factors, i.e., age, skin tone, latitude, sun exposure, and season of the year. In addition, hypovitaminosis D is a common problem in the city of São Paulo (Maeda et al. 2013). Therefore, without further evidence, it was not possible to confirm if the hypovitaminosis D was a comorbidity or a new phenotype.

Analysis of *SLC20A2* mRNA expression demonstrated only a small decrease in *SLC20A2* expression (~10 % decrease relative to controls) despite the proband carrying a premature transcription termination signal (stop codon). Zhang et al. (2013) performed similar experiments and described a decrease by ~30 % in *SLC20A2* expression in patients carrying the mutation. This might be due to compensation by the wild-type allele triggered by allelic loss. In our study, a stable expression of mutant *SLC20A2* mRNA or upregulation of the wild-type allele could account, on one hand, for the small differences we observed in our RT-qPCR. On the other hand, cDNA sequence reads suggest lower expression of the mutant allele (Fig. 2b), comparatively with the genomic DNA (Fig. 2a), suggesting an RNA-mediated decay, typically found in stop codon carriers.

*SLC20A2* and other genes associated with PFBC should also be screened for mutations in patients with non-idiopathic brain calcifications. The presence of hypovitaminosis D in the proband likely might exists as a comorbidity due to the absence of this condition in previous PFBC patients with *SLC20A2* mutations. However, it is necessary to document this finding and to be aware of occasional future reports.

Nicolas et al. (2014a) recently reported a de novo mutation in *PDGFB*, the second most frequent mutated gene in PFBC, in a patient with brain calcification and laryngeal dystonia. Together with our report, such findings reinforce the need to screen for mutations linked to PFBC even in cases with sporadic and atypical phenotype.

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**Conflict of interest** S.S., L.P., C.R., M.R.D.S., L.S., and J.R.M.O. work in public institution and did not receive any incentive from private companies. J.R.M.O. is the author of a book about familial brain calcification, edited by Nova Publishing (USA). J.F., L.P., M. K., R.R.L., M.F.O., N.J., and K.T. receive fellowships from graduate, undergraduate and post-graduate programs. The authors report no other disclosure and no conflict of interest directly linked to this report.

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SHORT REPORT

# Identification of partial *SLC20A2* deletions in primary brain calcification using whole-exome sequencing

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**Primary brain calcification (PBC) is a dominantly inherited calcifying disorder of the brain. *SLC20A2* loss-of-function variants account for the majority of families. Only one genomic deletion encompassing *SLC20A2* and six other genes has been reported. We performed whole-exome sequencing (WES) in 24 unrelated French patients with PBC, negatively screened for sequence variant in the known genes *SLC20A2*, *PDGFB*, *PDGFRB* and *XPR1*. We used the CANOES tool to detect copy number variations (CNVs). We detected two deletions of exon 2 of *SLC20A2* in two unrelated patients, which segregated with PBC in one family. We then reanalyzed the same series using a QMPSF assay including one amplicon in each exon of *SLC20A2* and detected two supplemental partial deletions in two patients: one deletion of exon 4 and one deletion of exons 4 and 5. These deletions were missed by the first screening step of CANOES but could finally be detected after readjustment of bioinformatic parameters and use of a genotyping step of CANOES. This study reports the first partial deletions of *SLC20A2* and strengthens its position as the major PBC-causative gene. It is possible to detect short CNVs from WES data, although the sensitivity of such tools should be evaluated in comparison with other methods.**
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## INTRODUCTION

Primary brain calcification (PBC), also known as primary familial brain calcification (OMIM 213600) or formerly Fahr's disease, is defined by the presence of microvascular calcification affecting at least the basal ganglia with no cause following an extensive clinical, biological, and imaging etiological assessment. Calcification carriers may exhibit diverse neurologic or psychiatric symptoms. Among them, the most frequent three categories are: psychiatric disturbances (mainly mood, psychotic or personality disorders), cognitive impairment (involving mainly memory and executive functions), and movement disorders (mainly extrapyramidal signs).[1] Patients may present other symptoms such as gait disorder, cerebellar syndrome, dysarthria, and rarely seizures. Cephalalgia and especially migraine are frequent circumstances allowing the identification of brain calcification.[1]

PBC is inherited as an autosomal dominant trait. In this context, causative variants have been identified in 4 genes: *SLC20A2* (OMIM 158378),[2] *PDGFRB* (OMIM 173410),[3] *PDGFB* (OMIM 190040),[4] and *XPR1* (OMIM 605237).[5] Two copy number variants (CNVs) were previously detected as PBC-causing: a partial deletion of *PDGFB* in one patient[6] and a deletion encompassing *SLC20A2* entirely and six other genes in one family.[7] *SLC20A2* and *XPR1* encode inorganic phosphate transporters, the importer SLC20A2 (also known as PiT2), and the exporter XPR1, respectively. Loss of function of either gene leads to microvascular mural and perivascular calcification involving vascular smooth muscle cells and pericytes. Loss-of-function variants of *PDGFB* or *PDGFRB* are responsible for an alteration of the blood–brain barrier (BBB). Whether the BBB alteration is the direct cause of microvascular calcification or there is other links with inorganic phosphate metabolism remains to be determined.[8] In our French series of PBC patients, >50% of probands or sporadic cases do not exhibit any causative variant in one of these genes after Sanger sequencing (unpublished data).

We performed whole-exome sequencing (WES) in 24 patients with PBC negatively screened for the four genes. Although WES was developed to detect single nucleotide variants and short indels, we can now take advantage from WES data to detect CNVs. We report here the results of CNV analysis of these four genes in these 24 WES.

## MATERIALS AND METHODS

### Patients

Patients were included following the previously described criteria.[9] In brief, probands exhibited (1) at least bilateral lenticular calcification, (2) the total calcification score using our own visual rating scale was above the age-specific threshold, and (3) an extensive etiological assessment was normal.

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Genetic analyses were performed after written, informed consent. This study was approved by our ethics committee. The entire coding sequence of *SLC20A2*, *PDGFB*, *PDGFRB*, and *XPR1* was assessed by Sanger sequencing.

We included for WES 24 patients with PBC (14 probands of unrelated families and 10 apparently sporadic cases) negatively screened for all four genes.

### Whole-exome sequencing

Exomes were captured using the Agilent Sureselect All Exons Human V5 Kit (Agilent technologies, Santa Clara, CA, USA). Final libraries were sequenced on a HiSeq2000 with paired ends, 100-bp reads. Reads were mapped to the 1000 Genomes GRCh37 build using BWA 0.7.5a.[10] Picard Tools 1.101 was used to flag duplicate reads. We applied GATK for indel realignment, base quality score recalibration and SNPs, and indels discovery using the Haplotype Caller across all samples simultaneously according to GATK 3.3 Best Practices recommendations.[11] The joint variant calling file was annotated using Annovar (date of version used: 2015/03/22 (http://www.openbioinformatics.org/annovar/).

### CNV calling

To detect CNVs from WES data, we used the CANOES suite.[12] Its algorithms are dedicated to the detection of CNVs using read-depth comparisons between different samples. In a first step, we calculated the read depth for each target using bedtools http://bedtools.readthedocs.io/en/latest/content/bedtools-suite.html). We merged close targets (<30 bp) to reduce the rate of false positives and removed all events localized in known regions of segmental duplication. As a second step, we used the genotype CNVs function of CANOES, which allows determining the probability of a specific event. The objective was to analyze each target intersecting any exon of *SLC20A2*, *PDGFB*, *PDGFRB*, and *XPR1* by considering each target as a putative independent event.

For exon numbering, we considered as exon 1, the first one from 5′ to 3′ in the cDNA following the given transcript. The three *SLC20A2* partial deletions were submitted to the LOVD database (http://databases.lovd.nl/shared/genes/SLC20A2/) with following variant IDs: 0000089490, 0000089491, and 0000089492.

### *SLC20A2* QMPSF assay

We designed a quantitative multiplex PCR of short fluorescent fragments (QMPSF) assay to confirm the presence of the deletion encompassing exon 2 of *SLC20A2* in two unrelated individuals and to screen all other patients. This assay included one control amplicon in *HMBS* and one amplicon in each exon of *SLC20A2* (NM_006749.4) plus one amplicon in alternative (non-coding) exon 1 (NM_001257180.1), which maps between non-coding exon 1 and (coding) exon 2 of transcript NM_006749.4 in the genome. Primers are available in Supplementary Table 1.

## RESULTS

Among the 24 WES of patients with PBC, mean depth of coverage was 135x (a mean of 98.97% of targeted bases were covered more than 15x). We extracted all variants mapping to *SLC20A2*, *PDGFB*, *PDGFRB*, and *XPR1* genes, and confirmed that no single nucleotide variant or short insertion or deletion probably affecting function had been missed by Sanger sequencing.

After exome-wide CNV calling using the CANOES tool, we detected a total of 273 events without any filtration on genes (11.4 per individual on average (range: 6–26), with a mean estimated size of 50.5 kb (range 0.099 kb–796.27 kb); 96.7% overlapped known CNVs from the database of genomic variants (http://dgv.tcag.ca/dgv/app/home). In total we identified 120 duplications and 153 deletions. Of these, 110 duplications and 134 deletions were identified only once in a single patient, that is, unique CNVs. We then focused on PBC genes and identified a heterozygous deletion of exon 2 of *SLC20A2* in two unrelated probands (ROU 1129 and EXT 383, Figures 1a and b, respectively). We confirmed the presence of both deletions by QMPSF and showed that this deletion only encompassed exon 2 and not exon 1 (NM_006749.4)), alternative exon 1 (NM_001257180.1), or exon 3. In family 1129, the presence of the deletion was confirmed in an affected brother and his affected daughter. DNA was neither available for other affected relatives from this family nor for relatives from family EXT 383. ROU 1129 and EXT 383 families both originate from the same region of France. However, pedigree information does not suggest that they are related and we confirmed the absence of close relatedness by estimating identity by descent through Plink software (Supplementary Note 1). Clinical and imaging findings are summarized in Table 1. A more detailed description of the phenotype is available in Supplementary Note 2.

The sensitivity of CNV detection has been estimated to be 74% in the first report of CANOES using WES data obtained with another capture kit.[12] With the aim to assess the presence or absence of CNV in each *SLC20A2* exon, we developed a QMPSF assay including one amplicon in each exon of *SLC20A2*. After assessment of the 22 remaining patients, we detected two new heterozygous deletions: one encompassing exon 4 only (EXT 434, Figure 1c) and the other one encompassing exons 4 and 5 (EXT 451, Figure 1d). The deletion of exon 4 was also found in the the proband's affected sister. DNA from relatives of the patient carrying the exons 4–5 deletion was not available. RNA from these patients was not available. None of the three deletions is reported in the database of genomic variants.

As 2 of the 4 deletions were missed by the first CNV screening of CANOES, we went back to WES data. We used another tool from the CANOES suite, called GenotypeCNVs, which is designed to perform targeted detection of CNVs, to test whether it allowed identifying all four deletions. The GenotypeCNVs tool allowed detecting all four deletions only after decreasing quality thresholds. However, this led to the detection of a false-positive duplication of exon 4 of *SLC20A2*.

## DISCUSSION

*SLC20A2* is the major gene causing PBC.[13] It is clearly demonstrated that heterozygous loss of *SLC20A2* function is sufficient to cause PBC.[2] However, only one genomic deletion has been published to date.[7] Among the seven genes deleted in this family, the *THAP1* gene also retained the attention as loss-of-function variants of this gene cause dystonia, which was a prominent feature associated with PBC in this family. We report here the first *SLC20A2* partial deletions causing PBC. Among 24 patients with genetically unexplained PBC, we found four (16.7%) *SLC20A2* partial deletions, suggesting that such events should be primarily assessed, together with *SLC20A2* coding sequence variants, during the genetic screening of PBC patients. In our series, *SLC20A2* causative variants, including sequence variants and CNVs, are found in 28.5% of unrelated probands (40% when focusing on familial cases).

Interestingly, almost all patients with a partial *SLC20A2* deletion exhibited the same radiological phenotype as patients carrying other loss-of-function variants, that is, calcification of lenticular, caudate nuclei, thalami, cerebellar hemispheres, and in particular the vermis and cortical sulci (Table 1,Supplementary Figure 1). All were symptomatic, which seems different from the 58% of symptomatic mutation carriers in our previous report.[1] Of note, in family ROU-1129, the grandmother developed cognitive decline only after the age of 86 (of unknown cause), and 2 affected relatives were asymptomatic by family interview. This suggests that the apparent high proportion of symptomatic patients in the present report could be an inclusion bias.

Exon 2 of *SLC20A2* is the first coding exon and contains the translation initiation ATG codon as well as very conserved amino acid residues. This deletion probably results in a loss of function. The deletion of exon 4 causes a frameshift and therefore a probable loss of function. A splice variant at the acceptor site of intron 4 has already

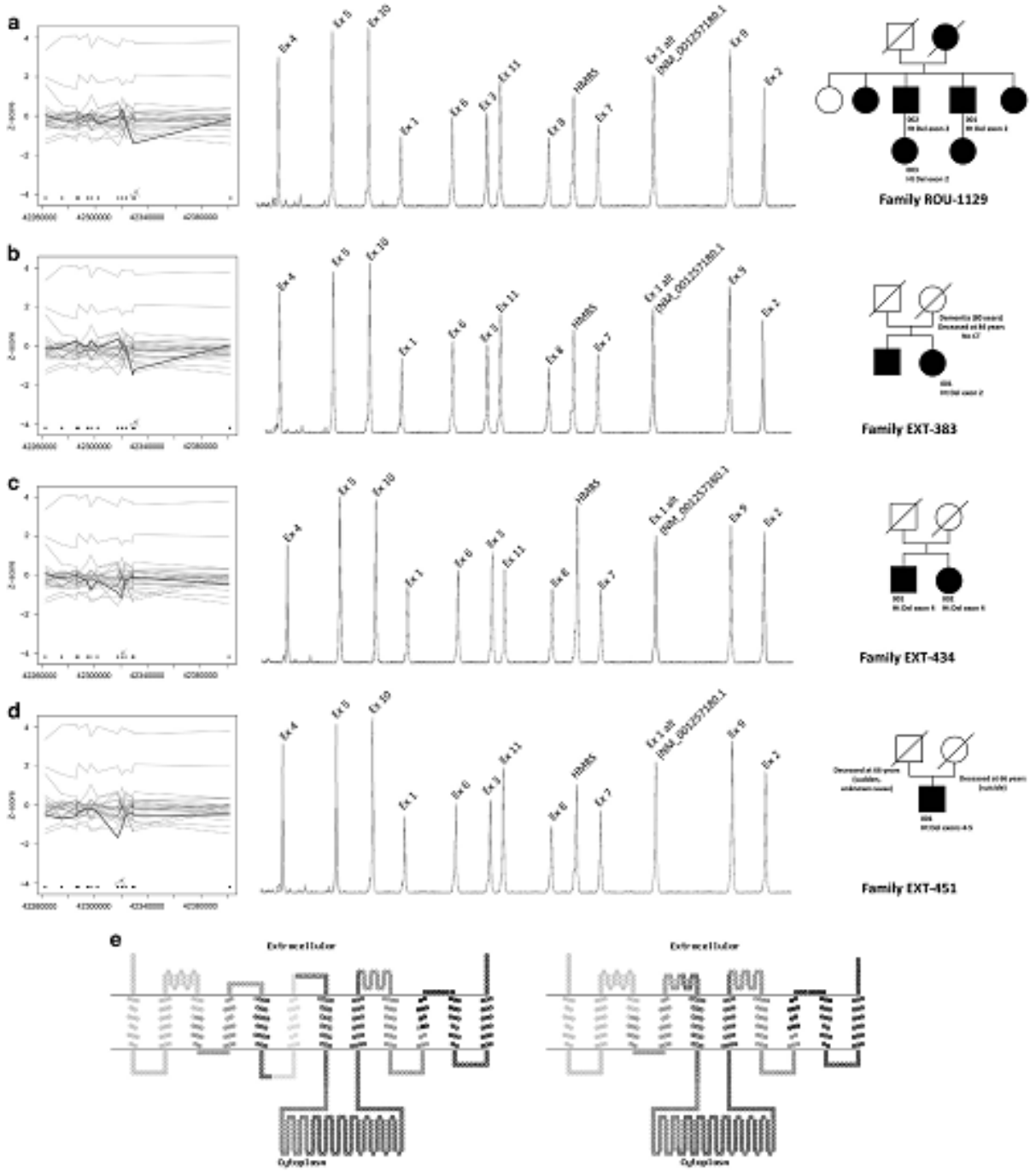


**Figure 1** Partial deletions of SLC20A2. (a–d) On the left part of the panels, Z score of read-depth comparison among the 24 exomes are represented along genomic positions on chromosome 8. Red lines represent each patient carrying a partial deletion. Black spots represent the targets of the capture kit. Arrows point the targets of exon 2 (a and b), exon 4 (c), and exons 4 and 5 (d). QMPSF electropherograms of the patients show partial deletions of *SLC20A2*; the patient electropherogram being in red, aligned to one control, in blue (middle part). The corresponding pedigrees are represented on the right part. (e) Schematic representation of the SLC20A2 protein as performed by the TOPO2 online software (http://www.sacs.ucsf.edu/TOPO2/) and according to ref. 14. Each color represents one different exon, the first coding exon being exon 2 (green). Monoexonic deletions of exons 2 or 4 are not represented as they are predicted to result in no protein or a frameshift; deletion of exons 4 and 5 is represented in the right part of the panel. A full color version of this figure is available at the *European Journal of Human Genetics* journal online.

been described in a French family[3] and could result in the same consequences as the genomic deletion of exon 4, that is, a total skipping of exon 4 in mRNA. However, RNA was not studied in the family with the splice variant and we cannot exclude that a cryptic acceptor site could be used. The deletion of exons 4–5 is in frame and results in the loss of two transmembrane domains (Figure 1e). Exon 4 is highly

**Table 1** Characteristics of patients with a partial deletion of *SLC20A2*

| ID | Sex | Age at first neuropsychiatric sign[a] | Age at last examination | Clinical summary | Calcification summary | TCS | Family history | Genetic status (SLC20A2) |
|---|---|---|---|---|---|---|---|---|
| ROU-1129-001 | M | 34 | 57 | - Migraine without aura<br>- Major depressive episode (34 years)<br>- Memory impairment, behavioral modification, dysarthria (55 years) | Le, Ca, Th, WM, Cb, Ve, Co | 57 | F | Heterozygous deletion of exon 2 c.(−265+1_−264−1)_(289+1_290-1)del (NM_001257180.1) |
| ROU-1129-002 | M | Unknown | 62 | - Alcohol abuse since adolescence, associated with epilepsy, cognitive impairment, gait disorder, and bradykinesia | Le, Ca, Th, Cb, Ve | 27 | F | Heterozygous deletion of exon 2 c.(-265+1_-264-1)_(289+1_290-1)del (NM_001257180.1) |
| ROU-1129-003 | F | Unknown | NA | - Migraine without aura<br>- History of mood disorder, unspecified (not examined by a neurologist) | At least Le | NA | F | Heterozygous deletion of exon 2 c.(-265+1_-264-1)_(289+1_290-1)del (NM_001257180.1) |
| EXT-383-001 | F | 57 | 61 | Progressive cognitive decline with dysexecutive syndrome (cognitive and behavioral), slight head tremor, intermittent dystonia of the limbs, pyramidal signs, gait disorder, dysphagia | Le, Ca, Th, WM, Cb, Ve, Co | 54 | F | Heterozygous deletion of exon 2 c.(-265+1_-264-1)_(289+1_290-1)del (NM_001257180.1) |
| EXT-434-001 | M | 20 | 63 | - Migraine without aura<br>- Intention tremor (20 years)<br>- Progressive dysarthria, gait disorder, mild static cerebellar syndrome with intention tremor and akinetic-hypertonic syndrome, cognitive dysexecutive syndrome (45 years) | Le, Ca, Th, WM, Cb, Ve, Co | 58 | F | Heterozygous deletion of exon 4 c.(430+1_431-1)_(516+1_517-1)del (NM_006749.4) |
| EXT-434-002 | F | 54 | 75 | Behavioral modifications, progressive cognitive decline leading to dementia, akinetic-hypertonic syndrome, tetrapyramidal syndrome, dysarthria and gait disorder | Le, Ca, Th, WM, Cb, Ve, Co | 58 | F | Heterozygous deletion of exon 4 c.(430+1_431-1)_(516+1_517-1)del (NM_006749.4) |
| EXT-451-001 | M | 66 | 76 | Recurrent major depressive episodes, cerebellar static syndrome | Le, Ca, Th, WM, Cb, Ve, Co | 51 | S | Heterozygous deletion of exons 4–5 c.(430+1_431-1)_(613+1_614-1)del (NM_006749.4) |

Abbreviations: Ca, caudate; Cb, cerebellar hemispheres; Co, cortex; F, female; Le, lenticular; M, male; NA, not applicable; TCS, total calcification score; Th, thalamus; Ve, vermis; WM, white matter.
[a]except for migraine.

conserved during evolution and it has been shown that an artificial deletion encompassing exon 5 and other exons resulted in decreased transport activity.[14] Taken together, this suggests that this deletion might also result in a loss of SLC20A2 function.

Although WES was not developed with the primary aim to detect CNVs, several tools take advantage from multiple comparisons of depth of coverage allowing the detection of deletions as short as 300 bp (eg, the deletions involving exon 2 of *SLC20A2*; size of the target in the capture kit: 316 bp). Exon 4 of *SLC20A2* is only 85 bp long (size of the target in the capture kit: 175 bp) and was detected by CANOES from WES data only after targeted genotyping. This suggests that the first step of CANOES, performed exome-wide, might be associated with good specificity but false negatives could be misleading. In presence of a reduced number of candidate genes, the genotyping step used with lower quality thresholds should allow reducing false negatives with the disadvantage of detecting false positives. Sensitivity and specificity of bioinformatics tools aiming at calling CNVs from WES data remain to be determined in comparison with other techniques allowing such a high resolution.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# Brain calcifications and *PCDH12* variants

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Supplemental data at Neurology.org/ng

## ABSTRACT

**Objective:** To assess the potential connection between PCDH12 and brain calcifications in a patient carrying a homozygous nonsense variant in *PCDH12* and in adult patients with brain calcifications.

**Methods:** We performed a CT scan in 1 child with a homozygous *PCDH12* nonsense variant. We screened DNA samples from 53 patients with primary familial brain calcification (PFBC) and 26 patients with brain calcification of unknown cause (BCUC).

**Results:** We identified brain calcifications in subcortical and perithalamic regions in the patient with a homozygous *PCDH12* nonsense variant. The calcification pattern was different from what has been observed in PFBC and more similar to what is described in in utero infections. In patients with PFBC or BCUC, we found no protein-truncating variant and 3 rare (minor allele frequency <0.001) *PCDH12* predicted damaging missense heterozygous variants in 3 unrelated patients, albeit with no segregation data available.

**Conclusions:** Brain calcifications should be added to the phenotypic spectrum associated with *PCDH12* biallelic loss of function, in the context of severe cerebral developmental abnormalities. A putative role for *PCDH12* variants remains to be determined in PFBC. Neurol Genet 2017;3: e166; doi: 10.1212/NXG.0000000000000166

## GLOSSARY

**BCUC** = brain calcification of unknown cause; **ExAC** = Exome Aggregation Consortium; **PFBC** = primary familial brain calcification.

A homozygous nonsense *PCDH12* variant has recently been reported in consanguineous families, where the affected children had congenital microcephaly, epilepsy, and profound global developmental disability.[1] Fetal MRI and USG showed dysplastic elongated masses in the midbrain-hypothalamus-optic tract area and hyperechogenic perithalamic foci. *PCDH12* encodes a protocadherin associated with membrane physical stability, adhesion, and vasculature maintenance and has recently been pointed out as a candidate gene for primary familial brain calcification (PFBC). PFBC is characterized by the presence of calcifications affecting primarily the basal ganglia, in the absence of secondary cause.[2] Clinical manifestations include movement disorders, cognitive impairment, psychiatric disturbances, and headache, most frequently beginning during adulthood.[2,3] Heterozygous variants causing autosomal dominant PFBC in up to 50% of the families were identified in 4 genes: *SLC20A2*, *PDGFRB*, *PDGFB*, and *XPR1*.[4–8] We previously searched for genes with a cerebral expression pattern similar to the PFBC major

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causative gene *SLC20A2* using the Allen Brain Atlas (brain-map.org/),[9,10] observing a higher *SLC20A2* expression in regions affected by calcifications in PFBC. *PCDH12* was singled out with the highest significant correlation,[10] and a follow-up analysis with additional brains still shows *PCDH12* as the most similar pattern to *SLC20A2*, even when compared with the other known PFBC causative genes (table e-1 at Neurology.org/ng).

To evaluate the potential link between *PCDH12* and brain calcifications, (1) we performed a CT scan in a patient reported to carry a homozygous nonsense *PCDH12* variant and (2) we screened DNA samples from patients with PFBC or brain calcifications of unknown cause (BCUC).

**METHODS** **CT imaging in *PCDH12* homozygous variant carriers.** In the original report, patients with symmetric intrauterine growth retardation, severe microcephaly, visual impairment, dystonia, epilepsy, and profound developmental disability were shown to carry a *PCDH12* c.995T>A, p.R839X homozygous variant.[1] This variant is considered to be pathogenic when carried at the homozygous state following the American College of Medical Genetics and Genomics and the Association for Molecular Pathology recommendations.[11] Brain imaging revealed midbrain hypothalamus dysplasia and significant periventricular and/or periventricular hyperechogenicity. Fetal USG and MRI did not enable to determine whether these foci are eventually calcifications. Therefore, we performed a brain CT scan in individual III-1, family B from the original pedigree.[1]

***PCDH12* screening in patients with brain calcification.** We included a total of 79 worldwide adult cases with brain calcifications that were referred to 5 centers of expertise, negatively screened for the known PFBC causative genes (supplemental data). Of these, 53 cases matched the clinical inclusion criteria for PFBC (detailed previously in reference 3). Briefly, these cases exhibited at least bilateral basal ganglia calcifications and no secondary cause. The remaining 26 patients were included on a neuropathologic basis if they presented moderate-to-severe basal ganglia calcifications. Note that calcifications also involved other brain regions in almost all cases and that other causes of brain calcifications could not be excluded in these patients, thereafter referred as having BCUC. All patients were screened for pathogenic variants by sequencing all coding exons of *PCDH12* (reference transcript: NM_016085.3). Bioinformatics predictions were performed using direct access to Polyphen2 HumDiv,[12] SIFT,[13] and Mutation Taster[14] tools, and the minor allele frequency (MAF) was checked at the Exome Aggregation Consortium (ExAC) website accessed in August 2016 (exac.broadinstitute.org/).[15] Detailed inclusion criteria and sequencing methods are provided in supplemental data.

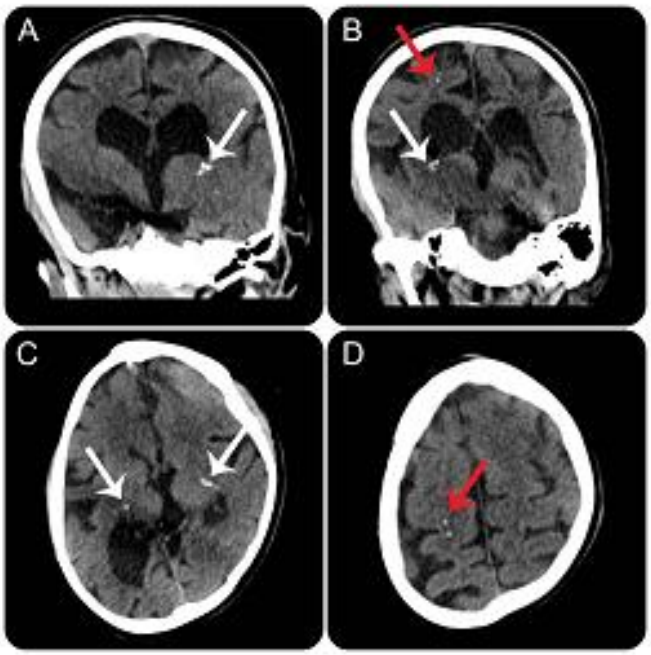
**Standard protocol approvals, registrations, and patient consents.** All patients provided written informed consent for genetic analyses.

**RESULTS** **CT of a *PCDH12* homozygous variant carrier.** CT is the reference imaging to identify brain calcification, so we used it to determine the nature of the hyperechogenic foci identified in a patient with a homozygous nonsense p.R839X *PCDH12* variant.[1] We identified spots of perithalamic calcification located in the posterior arms of the internal capsules and in juxtacortical right white matter (figure).

***PCDH12* screening in patients with brain calcification.** As we provided evidence that *PCDH12* biallelic loss of function is associated with brain calcification and given the high level of coexpression with the PFBC major causative gene *SLC20A2*, we next screened this gene in a group of patients with PFBC or BCUC. Among the 79 patients with PFBC or BCUC, we did not identify any protein-truncating variant (nonsense, splice site, or frameshift insertion/deletion). However, we detected 4 rare (MAF <0.001 in ExAC) heterozygous *PCDH12* missense variants in 4 unrelated patients: c.163C>G, p.(R55G); c.440G>T, p.(S147I); c.995T>A, p.(I332N); and c.3271G>A, p.(G1091S) (table 1). Three were predicted damaging by at least 1 in silico tool, while variant p.R55G was predicted benign by all 3 tools.

The c.440G>T, p.(S147I) variant had an MAF of 2.5e-05 in the ExAC database and was exclusively found in 3 individuals with the same ancestry as the patient (classified in ExAC as European non-Finnish). Two of the 3 in silico tools (Mutation Taster and Polyphen2 HumDiv, but not SIFT) predicted a damaging effect for this change to the protein

**Figure** **Brain CT imaging of a patient carrying the PCDH12 c.995T>A, p.R839X homozygous variant**




(A, B) Coronal sections. (C, D) Transversal sections. Spot calcifications affecting perithalamic regions (white arrows, A–C) and subcortical regions (red arrows, B, D).

**Table 1** Rare *PCDH12* variants identified in a series of 79 patients with PFBC or BCUC

| Location (Ghrc37) | cDNA change[a] | Protein change[a] | ExAC frequency[b] | SIFT prediction | Polyphen2 HumDiv prediction | Mutation Taster prediction | PhyloP |
|---|---|---|---|---|---|---|---|
| chr5:141337254 | c.163C>G | p.(R55G) | 6.1e-04 | Tolerated | Benign | Polymorphism | −1.01 |
| chr5:141336977 | c.440G>T | p.(S147I) | 2.5e-05 | Tolerated | Possibly damaging[c] | Disease causing[c] | 2.14[c] |
| chr5:141336422 | c.995T>A | p.(I332N) | 1e-04 | Deleterious[c] | Probably damaging[c] | Disease causing[c] | 4.48[c] |
| chr5:141325230 | c.3271G>A | p.(G1091S) | 3.3e-05 | Deleterious[c] | Probably damaging[c] | Disease causing[c] | 4.81[c] |

Abbreviations: BCUC = brain calcification of unknown cause; cDNA = complementary DNA; ExAC = Exome Aggregation Consortium; PFBC = primary familial brain calcification.
[a] Accession number: NM_016085.3.
[b] ExAC minor allele frequency assessed in August 2016.[15]
[c] Values are above each threshold.

function. DNA from relatives was not available for segregation analysis. This variant is located in the second cadherin tandem repeat domain (EC2) (NCBI accession cd11304) and, therefore, could affect homophilic adhesive behavior and calcium-dependent cell adhesion.[16]

The c.995T>A, p.(I332N) and c.3271G>A, p.(G1091S) variants are both predicted damaging by all 3 in silico tools. The p.I332N variant was reported with an overall MAF of 0.0001 in ExAC, found in 12 individuals of East Asian ancestry (the patient was born in Southeastern Asia) and 1 individual of European non-Finnish ancestry. The p.G1091S variant has an overall MAF of 3.3e-05, found in 1 individual of European non-Finnish ancestry (same as the patient) and 3 individuals of South Asian ancestry. DNA from relatives was not available for segregation analysis of any variant. Variant p.I332N is also located in a cadherin tandem repeat domain, namely EC3. However, p.G1091S variant is located in a highly conserved site in the cytoplasmic domain, which has a unique sequence among the cadherin family. Unlike the other cadherins, the cytoplasmic domain of PCDH12 does not interact with catenins, and it is involved in cellular processes other than cell junction, such as regulation of gene expression and signaling pathways.[17] Clinical details of all 3 predicted damaging variant carriers are provided in the supplemental data.

**DISCUSSION** We show here that a homozygous nonsense *PCDH12* variant, detected in patients with severe developmental delay and microcephaly,[1] is associated with brain calcifications. This feature should therefore be added to the phenotypic spectrum of this rare disorder. The pattern of calcifications is, however, different from the typical findings in PFBC, where calcifications always affect at least both pallidum,[3] and resembled to those observed in various neuroinfectious prenatal conditions, such as TORCH infections.[18] Brain calcification is a highly informative feature on brain imaging of children with neurodevelopmental disorders.[18] Although CT is the reference imaging tool for detecting and assessing calcifications, MRI is the primary imaging tool for the detection of all other brain abnormalities in the absence of radiation. T2* or susceptibility-weighted images increase the diagnostic performance of MRI for calcification compared with the other sequences. However, they can sometimes miss small calcifications, and they are still complementary with CT to describe precise shape and intensity and to definitely conclude on the differential identification with iron deposits.[19,20] In our patient, neither T2* nor susceptibility-weighted images were available.

In the original report, the efficiency of nonsense-mediated decay has been measured as 84%, suggesting a strong loss of function. The patients carrying the nonsense *PCDH12* variant in a homozygous state may still express little amount of the truncated protein, but no full-length PCDH12. This supports the hypothesis that loss of function of PCDH12 is the mechanism leading to the patient's phenotype, including brain calcification.

In a candidate gene approach, we searched for rare *PCDH12* variants in PFBC and BCUC patients and found no protein-truncating variants. Three heterozygous missense variants, predicted damaging by at least one of the tools, were identified in 2 patients with PFBC and 1 patient with BCUC. Given the fact that biallelic loss of *PCDH12* function leads to a severe neurodevelopmental phenotype, it is unlikely that these variants have a dominant-negative effect. However, as they are missense variants, their putative effect on protein function is hard to predict, and it remains possible that they are responsible for loss of function, gain of function, or have a neutral effect on protein function. The frequencies of theses variants in the patients' respective populations as estimated in ExAC are not inconsistent with a causative effect, as they are in the same frequency ranges as other disease-causing variants in *SLC20A2*.[8] Because neither segregation nor functional data are available, it is not possible to conclude about their pathogenicity at this stage.

Besides PFBC, brain calcifications can be detected in other numerous distinct conditions, such as

systemic phosphocalcic metabolism disorders of inherited or acquired cause, in utero or postnatal infections, interferonopathies, inborn errors of metabolism, and other rare inherited diseases.[21] Calcifications are believed to be related to increased type-I interferon response in both in utero viral infections and interferonopathies.[22] Several of these clinical presentations, including TORCH in utero infections and typical Aicardi-Goutières syndrome, are similar to the ones observed in the *PCDH12* homozygous carriers. In other conditions, mutations in *OCLN* and *JAM3* genes, encoding endothelial cell adhesion proteins, result in microangiopathy associated with calcifications.[18,23,24] Given the known function of PCDH12, we postulate that similar mechanisms could be associated with the calcifications observed in the *PCDH12* homozygous loss-of-function carriers.

PCDH12 is a protocadherin associated with membrane physical stability and adhesion.[25] A *Pcdh12* knockout mouse model revealed several age-independent vessel impairments, such as ramifications of medial elastic lamellae and increased inner diameter and circumferential mid-wall stress.[26] PCDH12 has been widely studied as a key-player cadherin involved in placental maintenance and also a preeclampsia biomarker; however, little is known about its involvement in brain physiology. It is conceivable that mutations in PCDH12 and SLC20A2, which share similar expression patterns in the brain, might lead to similar phenotypes. Of interest, *Slc20a2* knockout mice developed not only brain calcifications but also fetal growth restriction, lower birth viability, and placental calcification associated with thickened basement membranes.[27] In both mouse models, the placental phenotype and the vascular impairment are additional putative links between SLC20A2 and PCDH12, which deserve additional studies on mouse models.

*PCDH12* biallelic loss of function causes a severe neurodevelopmental phenotype associated with brain calcifications. Rare predicted damaging heterozygous *PCDH12* variants were identified in patients with PFBC or BCUC here, but whether they are associated with brain calcification or not remains to be determined. To address this question, follow-up studies will be necessary including screening other series, assessing the segregation of rare variants and functional consequences.

## AUTHOR CONTRIBUTIONS

Collection and interpretation of data: Gaël Nicolas, Monica Sanchez-Contreras, Eliana Marisa Ramos, Roberta R. Lemos, Joana Ferreira, Denis Moura, Maria J. Sobrido, Anne-Claire Richard, Alma Rosa Lopez, Andrea Legati, Jean-François Deleuze, Anne Boland, Olivier Quenez, Pierre Krystkowiak, Pascal Favrole, Daniel H. Geschwind, Adi Aran, Raoul Segel, Ephrat Levy-Lahad, Dennis W. Dickson, Giovanni Coppola, Rosa Rademakers, and João R.M. de Oliveira. Manuscript draft: Gaël Nicolas, Monica Sanchez-Contreras, Eliana Marisa Ramos, Roberta R. Lemos, and João R.M. de Oliveira. Critical revision: Gaël Nicolas, Monica Sanchez-Contreras, Eliana Marisa Ramos, Roberta R. Lemos, João R.M. de Oliveira, and Giovanni Coppola. Study design and supervision: João R.M. de Oliveira, Gaël Nicolas, Giovanni Coppola, and Rosa Rademakers.

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# 5. GENERAL RESULTS AND DISCUSSION

## 5.1. SEQUENCING OF PBC CANDIDATE GENES

The coding region of the four genes associated with PBC was sequenced in 15 Brazilian patients (6 male and 9 female). This comprises *SLC20A2* exons 2 to 11 (NM_006749.4, MIM:158378), *XPR1* exons 1 to 15 (NM_004736.3, MIM:605237), *PDGFB* exons 1 to 6 (NM_002608.2, MIM:190040) and *PDGFRB* exons 2 to 23 (NM_002609.3, MIM:173410). The whole set of coding reference sequence variants found by this work is resumed in Table 1.

Regarding these genes, only one novel variant was found, which was the first *SLC20A2* de novo mutation confirmed in a patient with brain calcifications, migraine and hypovitaminosis D (NM_006749.4:c.1158C>G, NP_006740.1: p.Y386*; for details of this case please see Ferreira et al. 2014 at section 7 of this thesis). This non-sense variation codes a premature stop codon (PTC), resulting in a putative truncated protein with 385 amino acids instead of 652. At the time of this finding, we suggested that the hypovitaminosis D was most likely a comorbidity because this condition has not been observed in previous PBC patients with *SLC20A2* variants, and that occasional future reports might enlighten this question. In fact, another non-sense mutation in the same position (NM_006749.4:c.1158C>A, NP_006740.1: p.Y386*) was found in two unrelated French PBC patients in 2015, both with normal vitamin D levels and at least one of them with migraine (Gaël NICOLAS, personal communication), thus enforcing the comorbidity aspect of the hypovitaminosis D in the Brazilian patient.

*SLC20A2* coding region and exon/intron boundaries showed the least number of variation among all eight genes screened. Besides the de novo nonsense mutation above mentioned, only three other mutations were observed, each in a different patient: a synonymous mutation in exon 3 (c.345G>A, p.T115, rs34124953), an intronic mutation between exons 5 and 6 (c.614-93G>A, rs188779206), and a missense mutation in exon 7 (c.910G>A, p.Gly304Ser, rs73675069). Although there is no MAF available for the intronic variant, both coding variants have a MAF of ~5%, which is higher than the cutoff for rare variants and corresponds to a polymorphism frequency. These three variants have been predicted in general as having no effect in *SLC20A2* function by Provean, SIFT, PolyPhen2 and/or MutationTaster2 (MT) online prediction tools.

Surprisingly, only the synonymous mutation in exon 3 had a controversial prediction as disease-causing by MutationTaster2 while PolyPhen2 and Provean/SIFT considered it as benign and tolerated, respectively. This contradiction might be due to MT has been designed specifically to aid the identification of rare variants with severe impact, and it is more stringent than the other tools when it automatically assigns a specific model by mutation type[9]. There is one prediction model specific for intronic and synonymous mutations, one for single amino acid changes and one for complex amino acid alterations such as mutations introducing a premature stop codon. When the four above-mentioned online tools were compared regarding single amino acid changes, MT was shown to be the most accurate according to Schwarz et al. (2014). MT is also presents higher sensitivity then specificity, which means that it generally rather risk false positives than miss any true positives.

For *XPR1* gene, on the other hand, variants were detected in all 8 screened patients – the others had already been analyzed and excluded for *XPR1* mutations by Legati et al. (2015). Three patients presented one different synonymous mutations each: one in exon 4 (NM_004736.3:c.408T>C, NP_004727.2:p.Ser136, rs12078050), one in exon 9 (c.1128A>G, p.Lys376, rs35706835), and one in exon 10 (c.1158C>T, p.Phe386, rs61742073). All were predicted to be polymorphic and to have no effects in function. The majority of the variants were intronic, and although some were predicted to change splicing site, none was predicted as disease-causing and most were polymorphisms with MAF above the 2% cutoff.

Regarding de platelet-derived growth factor family, the receptors' genes presented more coding variants predicted to be polymorphisms than the binding-factors' genes. Both the PBC-associated *PDGFRB* and its homologue *PDGFRA* presented numerous synonymous and missense mutations shared among two or more patients, reflecting the high MAF of these variants. Among the binding-factors, the PBC-associated *PDGFB* was the most conserved with no coding mutations detected and presented only four intronic, non-affecting variants with high MAF observed in most patients. *PDGFA* presented three intronic variants with the same characteristics, and only two patients bearing one coding, synonymous variant (NM_002607.5:c.207T>C; NP_002598.4:p.His69, rs1129401) with a high MAF of ~25%. For *PDGFC*, only two variants were found, one synonymous (NM_016205.2: c.1032A>G, NP_057289.1:p.Gly344, rs3815861) observed in four patients and an intronic detected in only one patient, both with high MAF and non-affecting predictions.

PDGFD was the less conserved among the three binding-factors' genes. The sequencing of the 14 patients revealed three coding variants located in the terminal exon 7 and seven intronic mutations dispersed through exons 2, 4, 5 and 7. The synonymous polymorphism (NM_025208.4:c.1080T>C, NP_079484.1:p.Cys360, rs10791649) reflected its MAF of 47% and was observed in 8 patients. One missense mutation, in the exon 4, was detected in one patient (c.568A>G, p.Ile190Val, rs35045740) and had a MAF of 3%. However, the other missense variant, located in the terminal exon 7 (c.1081G>A, p.Asp361Asn, rs146343067), also detected in only one patient, had no MAF available in the online databases and was predicted as affecting gene function: disease causing by MT, and problably or possibly damaging by PolyPhen2 (HumVar and HumDiv algorithms, respectively). According to Hoch et al. (2003), the PDGF-D peptide only forms homodimers PDGF-DD and only binds to a receptor with a PDGFR-β unity, either the homodimer PDGFR-ββ or the heterodimer PDGFR-αβ. In fact, Bergsten et al. (2001) affirmed that PDGF-DD homodimer needs the formation of four disulfide bonds, one of them envolves cysteine in position 362. The amino acid change in position 361 resultant from this variation might cause the lost of this disulfide bond and affect negatively PDGF-DD function, as predicted by MT analysis.

Interestingly, the patient bearing this variation differed of the others in presenting no other mutation in the *PDGFD* coding region. Consistently, *PDGFRB* gene sequence in this patient presented few variants: two synonymous (rs246395 and rs246388) and one intronic (rs246391) polymorphisms, all with MAF >20%. The fact that *PDGFD* final product interacts only with the final product of a PBC-associated gene might point to a possible role in brain calcification formation.

Primary brain calcifications seems to segregate by the patient's mother family side. Not only his mother has CT scan positive for calcifications in the basal ganglia, but also one male and one female cousins as well as an aunt. Sequencing the region of this mutation in the family is needed in order to confirm or not the role of *PDGFD* in the formation of PBC.

### 5.2. *SLC20A2* QMPSF

QMPSF is a method very sensitive in the detection of duplications and deletions, based on the simultaneous amplification of multiple DNA fragments by fluorescent-labeled primer pairs used under quantitative conditions, as defined by Charbonnier et al.

(2000). After the screening of twenty-two Brazilian patients with brain calcifications for *SLC20A2* exons' deletions and duplications by QMPSF, only one patient was positive for a partial copy number variation, showing a duplication of the terminal exon 11 (Figure 2).

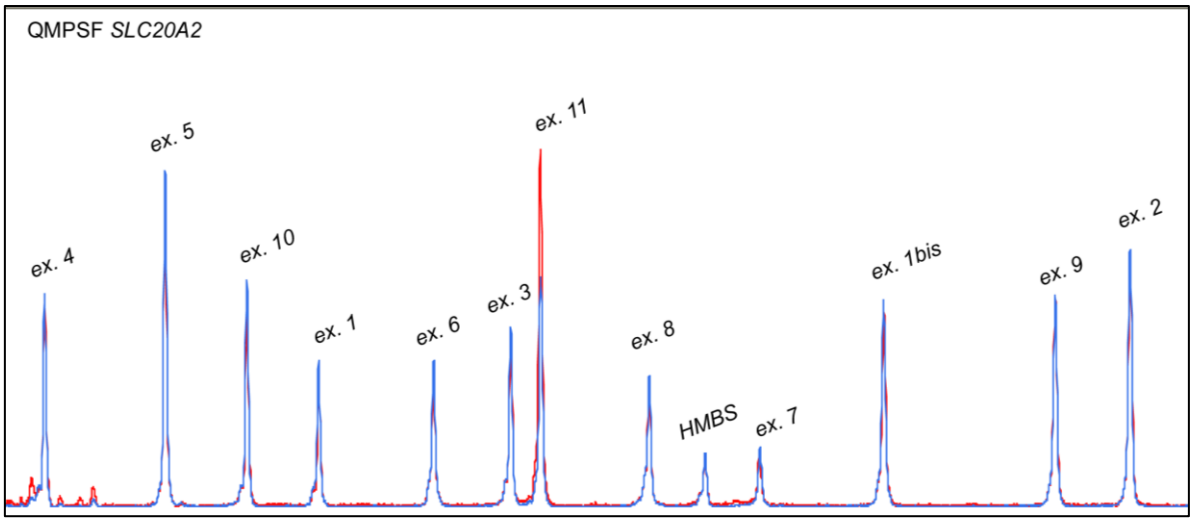
This patient is 31 years old, male, has hipovitaminosis D and hyperparathyroidism. Since hyperparathyroidism is a known cause of brain calcifications according to Manyam (2005), the basal ganglia calcification observed in this case could be secondary to this condition. Therefore, this is probably not a PBC case.

Exon duplications in genes related to neurogenerative diseases have been reported, such as *SNCA* exons 3 and 4 for Parkinson's disease reported by Ibáñez et al. (2004), and *APP* exons 1, 7 and 18 for autosomal dominant early-onset Alzheimer disease as reported by Rovelet-Lecrux et al. (2006). Considering that the duplication is disrupting the gene and consequently affecting negatively its function, the gene breaking point was searched. The first step was to better estimate the extension of this duplication, and a second QMPSF reaction was designed. Three target regions near *SLC20A2* exon 11 were added: *VDAC3*, the gene nearest to *SLC20A2* exon 11; *VDAC3* and *SLC20A2* intergenic region; and *SLC20A2* intron 10 (located between exons 10 and 11). Again, only *SLC20A2* exon 11 was duplicated (Figure 3). This narrowed the searching area to a 5kb region between *SLC20A2* intron 10 amplicon and exon 11.

Assuming that the duplication is *in tandem* and inverted, new sequencing primers were designed, all in the foward sense. They were used in different combinations to perform PCR in DNA samples from both the patient and a control. Hypothetically, the PCR would only work for breaking point region, because then one primer would anneal to the inverted sequence and play the role of a reverse primer. Although PCR agarose gel bands exclusive for the patient have been obtained, their sequencing was inconclusive.

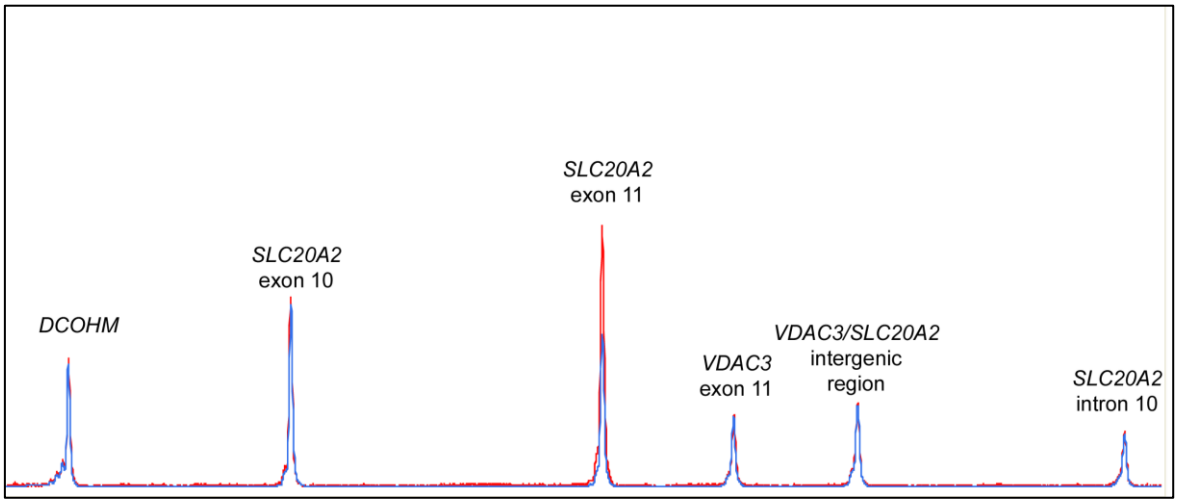
Recently, we extracted DNA from new blood samples not only from this patient but also from his parents. A second *SLC20A2* QMPSF screening in this new DNA samples will permit to confirm the duplication in the patient and to verify whether it is segregating in the family.

Figure 2. QMPSF electropherogram profile for *SLC20A2* gene in a patient with exon 11 duplication. The profiles are shown in red for the patient and in blue for the control DNA.




Source: The Author (2016)

Figure 3. QMPSF electropherogram profile specific for the investigation of SLC20A2 exon 11 duplication. In this case, the control gene utilized was *DCOHM*. The profiles are shown in red for the patient and in blue for the control DNA.




Source: The Author (2016)

Table 1. Coding variants of PBC associated and candidate genes detected by Sanger Sequencing in Brazilian patients. The global minor allele frequency (MAF) was obtained from dbSNP online database; the total of alleles' sequences is shown in parenthesis. The Local Frequency shows the number of patients bearing the variant in relation to the number of patients screened for a given gene.

| Gene | Variants[a] | Exon | Bioinformatic Prediction | MAF | Local Frequency |
|---|---|---|---|---|---|
| *SLC20A2* | c.345G>A<br>p.T115<br>rs34124953 | 3 | Controversial[b] | 0.5%<br>(26) | 1/15 |
| | c.910G>A<br>p.Gly304Ser<br>rs73675069 | 7 | No effect | 5.2%<br>(261) | 1/15 |
| | c.1158C>G<br>p.Tyr386* | 8 | Disease causing | N/A[c] | 1/15 |
| *XPR1* | c.408T>C<br>p.Ser136<br>rs12078050 | 4 | No effect | 5.1%<br>(258) | 1/8 |
| | c.1128A>G<br>p.Lys376<br>rs35706835 | 9 | No effect | 1.3%<br>(65) | 1/8 |
| | c.1158C>T<br>p.Phe386<br>rs61742073 | 10 | No effect | 5.1%<br>(258) | 1/8 |
| *PDGFRB* | c.85A>T<br>p.Ile29Phe<br>rs17110944 | 3 | No effect | 9.8%<br>(491) | 1/14 |
| | c.1033C>T<br>p.Pro345Ser<br>rs2229558 | 7 | No effect | 45.7%<br>(496) | 1/14 |
| | c.1149G>C<br>p.Leu383<br>rs2228439 | 8 | Controversial[b] | 0.5%<br>(24) | 1/14 |
| | c.1453G>A<br>p.Glu485Lys<br>rs41287110 | 10 | No effect | 1.1%<br>(54) | 3/14 |
| | c.1391C>T<br>p.Thr464Met<br>rs74943037 | 10 | No effect | 2.8%<br>(138) | 1/14 |

[a] RefSeq: NM_006749.4 and NP_006740.1 (*SLC20A2*), NM_004736.3 and NP_004727.2 (*XPR1*), NM_002609.3 and NP_002600.1 (*PDGFRB*).
[b] Considered disease causing only by MutationTaster2.
[c] N/A: Not available.

Table 1. (Continuation).

| Gene | Variants[a] | Exon | Bioinformatic Prediction | MAF | Local Frequency |
|---|---|---|---|---|---|
| *PDGFRB* | c.1854G>A p.Thr618 rs56072663 | 13 | No effect | 1.8% (92) | 1/14 |
| | c.2601A>G p.Leu867 rs246395 | 19 | No effect | 23.6% (1184) | 10/14 |
| | c.3090C>T p.Pro1030 rs2228440 | 22 | No effect | 6.2% (310) | 3/14 |
| | c.3252A>G p.Pro1084 rs246388 | 23 | No effect | 28.3% (1429) | 11/14 |
| | c.3270G>A p.Pro1090 rs183852315 | 23 | No effect | N/A[c] | 1/14 |
| *PDGFRA* | c.201C>T p.Ser67 rs35805947 | 3 | No effect | 0.3% (14) | 1/14 |
| | c.612T>C Asn204 rs2229307 | 4 | No effect | 23.6% (1183) | 3/14 |
| | c.939T>G p.Gly313 rs4358459 | 7 | No effect | 23.4% (1173) | 4/14 |
| | c.1432T>C p.Ser478Pro rs35597368 | 10 | No effect | 19.9% (998) | 3/14 |
| | c.1701A>G p.Pro567 rs1873778 | 12 | No effect | 4.2% (212) | 2/14 |
| | c.1809G>A p.Ala603 rs10028020 | 13 | No effect | 22.8% (1142) | 2/14 |
| | c.2472C>T p.Val824 rs2228230 | 18 | No effect | 24.0% (1204) | 2/14 |

[a] RefSeq: NM_002609.3 and NP_002600.1 (*PDGFRB*), NM_006206.4 and NP_006197.1 (PDGFRA)

Table 1. (Continuation) .

| Gene | Variants[a] | Exon | Bioinformatic Prediction | MAF | Local Frequency |
|---|---|---|---|---|---|
| *PDGFRA* | c.3222T>C<br>p.Asp1074<br>rs7685117 | 23 | No effect | 0.3%<br>(14) | 2/14 |
| *PDGFA* | c.207T>C<br>p.His69<br>rs1129401 | 3 | No effect | 24.8%<br>(1244) | 2/14 |
| *PDGFC* | c.1032A>G<br>p.Gly344<br>rs3815861 | 7 | No effect | 18.1%<br>(394) | 5/14 |
| *PDGFD* | c.568A>G<br>p.Ile190Val<br>rs35045740 | 4 | No effect | 3.3%<br>(165) | 1/14 |
| | c.1080T>G<br>p.Cys360<br>rs10791649 | 7 | No effect | 46.1%<br>(2311) | 8/14 |
| | c.1081G>A<br>p.Asp361Asn<br>rs146343067 | 7 | Disease causing | N/A[c] | 1/14 |

[a] RefSeq: NP_006197.1 (PDGFRA), NM_002607.5 and NP_002598.4 (*PDGFA)*, NM_016205.2 and NP_057289.1 (*PDGFC*), NM_025208.4 and NP_079484.1 (*PDGFD*)
[c] N/A: Not available
Source: The Author (2016).

## 6. CONCLUSIONS

PBC is a rare disease, often silent during its early stages, with heterogeneous signals and symptoms which hinders its diagnosis. Non-invasive and highly sensitive techniques, such as the utilized in this thesis, will permit an earlier diagnosis at least for familial cases.

The nonsense variant found in *SLC20A2* exon 8 was the first *de novo* mutation described for this gene. Together the two other French cases in the same position, it not only reinforces the role of this gene but also shows the importance of this exon for PBC onset and diagnosis.

Partial CNVs, even deletions or duplications of only one exon, also plays a causative role in PBC. Techniques efficient in their detection should be included in the initial steps of standard screening for PBC genetic variants along with Sanger sequencing of candidate genes' coding region.

One of these techniques is the QMPSF reaction designed here for *SLC20A2*, whose have been successful in screening deletions and duplications in multiplex. Thus, it is now possible to screen all *SLC20A2* exons simultaneously in one QMPSF reaction. The design of QMPSF reactions for the other PBC-genes might contribute to achieve a more time and cost efficient diagnosis.

The analyses performed in this thesis shows the relevance of both the search of new candidate genes and the screening of already associated genes. Therefore, investments in both fronts will aid a better comprehension of PBC manisfestation.

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